A STUDY OF EPIDERMAL FIBRILS.

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PLATE XXIII.

When the epidermis* is appropriately stained delicate fibrils can be seen in the stratum filamentosum. These fibrils go out from the protoplasm of the cells in different directions and across the intercellular lymph spaces to enter neighboring cells, so that all the cells of this layer are bound together by a delicate fibre system. The departure of the fibrils from the cells gives them the appearance of a thorn-like border whence they have derived the name "prickle" cells. These fibrils, which have been termed intercellular bridges, present in their middle part a slight enlargement, which is known as the Ranvier-Bizzozero nodule. They are not confined wholly to the stratum filamentosum but also may be seen joining together many of the basement cells. In addition to these connecting fibrils there may be seen in the stratum germinativum longer, thicker, more deeply staining protoplasmic fibrils, which run parallel to the long axis of the

* In the human epidermis seven layers are recognized by Ehrmann and Fick (*Einführung in das mikroskopische Studium der normalen und kranken Haut.* Wien, 1905). From below upward they are designated as follows: 1. Stratum germinativum. 2. Stratum filamentosum. 3. Stratum granulosum. 4. Stratum intermedium. 5. Stratum lucidum. 6. Stratum corneum. 7. Stratum disjunctum. The lower cells (stratum germinativum) are placed perpendicular to the corium; they have large, oval, vesicular nuclei with considerable chromatin; the shape of the cells varies from that of a cube to that of a slender high cylinder; they are closer together than in the layer above but are separated by narrow lymph channels. This layer passes insensibly into the stratum filamentosum, the cells becoming larger and more polygonal in shape; the nuclei are larger, rounder, and do not stain so deeply as the nuclei of the basal cell row. The channels between the cells are wider than below. Above this layer the cells are flatter and show granules of kerato-hyalin and finally become fused together and disintegrate. They need not be further described as only the lower two layers are to be considered in this paper.

cells. These fibrils frequently are distinctly spiral in form. They may follow the contour of the nucleus and be wholly within the cell or they may lie at the edge of the cell or in the lymph space between the cells. They appear to be wholly distinct from the fibrils first described. In the normal epidermis these latter fibrils are few and they may be wholly absent in many sections. It is in certain pathological conditions, however, that these fibrils assume importance and offer the basis for the present study.

Among the numerous articles that have appeared concerning these fibrils only the more important need be noted.

In 1863, Schroen[1] described in the rete Malpighii of the epidermis "scalariform striæ," which he considered a part of the cell membrane "with pores to let in nourishment." Max Schultze,[2] in 1864, observed "projections" from the cells of the mesial zone of the epidermis and was the first to apply to these cells the name of "prickle (*Stachel*) cells." He believed that these spines "gripped one another as bristles do when two brushes are pressed together." An important contribution was made by Bizzozero[3] (1871), who first noted the minute thickenings or nodules on these spines. These nodes he considered to be the point of union of two fibrils "welded together end on end." Ranvier[4] (1879) also studied these fibrils and said, "The cells of the rete Malpighii are not individual but are united by protoplasmic filaments. Each of these filaments is the result of the union of two filaments end on end, and the nodule is the result of their union." The nodule Ranvier further considered to be an elastic organ permitting easy enlargement of spaces destined for circulation of lymph between the cells. In 1882, Ranvier[5] published a second article, in which he described these fibrils as "filaments of union absolutely continuous," instead of being two fibrils united end on end.

Up to this time all discussion as to epithelial fibres had centred about these protoplasmic "bridges" or "spines" of the rete Malpighii. In 1889, Herxheimer,[6] by aid of the then new Weigert fibrin stain, described a new variety of fibril which he found running parallel to the long axis of the cells of the lower layers of the epithelium (stratum germinativum). He did not reach a conclusion concerning the significance of these fibrils, but Eddowes,[7] working in Unna's laboratory, came to the conclusion that these Herxheimer fibrils were only fibrin-filled lymph spaces. A year later, Kromayer[8] turned his attention to these fibrils. He used a modification of the Weigert stain which apparently resulted in a better picture than Herxheimer had obtained. Kromayer considered these fibres to be true protoplasmic fibrils, and not artifacts. In addition to the Herxheimer fibrils he also described a special fibril (*Haftfaser*) extending into the corium and possibly serving to join the corium to the epidermis. Some of the fibrils Kromayer described as having a corkscrew shape.

Kromayer's views were strongly opposed by various investigators. Beneke[9] believed these fibrils to be only a part of the cell membrane. Schütz[10]

considered Kromayer's fibres to be only the well-known "Ranvier" fibres altered by the staining method used. This author also considered Herxheimer's fibres to be an artifact. In the same year Herxheimer and Müller,[11] by aid of Weigert's neuroglia stain, reached the conclusion that these basal fibres represented the cuticular membrane of the cells and that the fine protoplasmic fibrils might be differentiated protoplasm.

Rabl[12] (1897) investigated these fibrils and considered the spiral form of the Kromayer fibrils an artifact. Rabl believed that the fibrils were formed by the coalescence of numerous finer fibrils which through shrinkage became a compact, broad fibre. Kromayer,[13] in a later paper, defended the "corkscrew" fibre, which he considered a distinct fibril in no way to be considered a part of the cell membrane. Herxheimer[14] also devoted a later paper to these fibrils which he described as being produced from a certain specific part of the cell protoplasm.

Unna[15], who has contributed several articles on these fibrils, in a recent paper devoted in great part to a consideration of a new stain gives us some excellent pictures of the various fibrils of the epidermis. Unna believes that the Herxheimer and similar fibrils are a part of the general fibre system.

It will be seen from the foregoing that there is still considerable dispute regarding the nature and significance of these fibrils. Although at first some observers considered the basement fibrils as artifacts, the later observers agree that they are true fibrils. The spiral form, however, that certain fibres assume is considered by some artificial. There is a dispute as to whether the fibres are a product of the protoplasm of the epithelial cells. By some they are considered merely the differentiated cuticular membrane, by others a condensation of the fibrillar ground substance of the cells. Moreover, it is a disputed question whether these fibrils are all a part of one general system, or whether the various fibrils are to be considered separate and distinct. While the fibrils of the basement layer are described in conditions incident to epithelial proliferation there seems to be no stress laid on the relation of fibre production to cellular activity.

The different views regarding these fibrils may be accounted for, in part at least, by the somewhat complicated technic by which they have been studied. Previously the Weigert fibrin stain or some of its modifications, such as the Kromayer stain, or Unna's method have been used for their demonstration. All these methods are, however, extremely complicated, and admit

of good results only after considerable practice. Fischel[16] has recently described a modification of the Kromayer stain that shortens the process somewhat, but is still far from simple.

For the demonstration of these fibrils the phosphotungstic acid hematoxylin stain,* as described by Mallory[17] for fibroglia fibrils, was used. Two methods were employed in the examination of tissues, as follows:

Method A.—1. Fix in Zenker's fluid; small pieces of skin are stretched out on filter paper and placed in the fluid immediately after excision.

2. Transfer celloidin or paraffin sections from water to a one-fourth per cent. aqueous solution of permanganate of potassium for from five to ten minutes; wash in water; place in a five per cent. solution of oxalic acid for from fifteen to thirty minutes; then wash thoroughly in water.

3. Stain in phosphotungstic acid hematoxylin for from twelve to twenty-four hours; wash quickly in water; dehydrate in alcohol; clear in xylol, and mount in xylol balsam.

Method B.—1. Fix freshly excised pieces in four per cent. formaldehyde (ten per cent. formalin). 2. Stain frozen sections, without mordanting, in phosphotungstic acid hematoxylin for from thirty minutes to four hours.

3. Immerse in water, alcohol, xylol, and xylol balsam as before.

For routine study the Zenker-fixed material was used and in most cases both paraffin and celloidin sections made. By this method the epidermal fibrils are stained a deep blue. The intercellular connective-tissue fibrils of the corium are pale brownish red. The nuclei of the cells are stained blue.

In addition to the sections that were prepared as above, frozen sections were made by Method B and carried along for the purpose of comparison. These sections were a considerable help in the study. When properly stained by this method everything in the section with the exception of the epidermal fibrils is a pale brownish red, while the fibrils are a deep blue. This gives a contrast that is not obtained by Method A. The objection to this method is, however, that at times there is inadequate fixation, and therefore loss of fibrillar structure, which the Zenker-

* It is necessary to use a well ripened stain to get good results. Mallory suggests the addition of an oxidizing agent, such as a small amount of peroxide of hydrogen, if the solution does not stain. In this work a stain somewhat over a year old was used. The staining time, as given here, may have to be prolonged if a less intense stain is used.

fixed material does not suffer. I can account for this only by the fact that the quality of commercial formaldehyde solutions cannot be depended on. If one could feel certain of the fixation, the simplicity of this technic, the quicker action of the stain, and the better contrast in the sections, would make it commend able. It is at any rate essentially practical for a rapid demonstration of epidermal fibrils in freshly excised tumors of the skin.

In the course of variola diagnosis in which both the above methods were employed, these fibrils of the epidermis were first noted. This simple technic, which seemed to obviate as far as possible all artifacts and to permit a fairly strict construction of the pictures obtained, seemed so well adapted to their study that a systematic effort was made to obtain various skin sections, which might offer some points of interest to our knowledge of the epidermal fibre system. In common with many other histologic and pathologic problems it was assumed that a preliminary study of these fibrils in animals would be of service in their final interpretation. Herxheimer (*loc. cit.*) has stated that in the animals which he examined (rabbit, rat, and guinea-pig) the long fibrils of the basal cells were wanting.

By permission of Mr. Frank Hagenbeck and by assistance of Dr. R. B. H. Gradwohl, to whom I wish to express my indebtedness, I was enabled to obtain skin sections from a number of the animals in the Hagenbeck zoological collection. Sections of skin from twelve animals were examined.

In regard to the cells of the epidermis of these animals it may be said that usually the different layers as described in human epidermis can be made out. There may be, however, a poor development of any one of the layers. Some animals show a sudden transition from stratum germinativum to stratum granulosum, with little development of the polygonal cells of the stratum filamentosum. Others show the polygonal cells extending to the corium with only occasional suggestion of cells of a columnar type in the stratum germinativum. In regard to the fibres, however, there is a considerable divergence, especially in certain of the animals, from the picture seen in human epidermis. In

all the animals excellent pictures are obtained of the connecting fibrils of the stratum filamentosum. They appear more prominently than they do in similarly stained sections of human epidermis. It is in the stratum germinativum that the difference between animal and human skin and among the skins of the various animals examined is to be noted. This difference is in the development of the fibrils of the basement layer and therefore a separate description of these fibrils follows:

Kangaroo (*Macropus major*).—Only an occasional fibril is seen, extending from the corium to the upper pole of the nucleus in one of the lower rows of cells. The cells of the basement row are not distinctly columnar in this animal.

Puma (*Felis concolor*).—Basement fibrils are rarely seen. Epithelial cells are flattened and the epidermis is only two or three cell layers deep. This skin was taken from a young animal.

Tapir (*Tapirus indicus*).—There is better development of the cells than above, and quite a number of short, sometimes wavy, fibrils are present in and between cells of the basement row.

Fat-tailed sheep (*Ovis aries steatopyga*).—The epidermis here is poorly developed, but an occasional wavy fibril may be seen, usually lying at the edge of the cell.

Water deer (*Hydropotes inermis*.).—Several fields in the section show groups of the fibrils both within and at the edge of the lower cells. Occasionally a fibril extends into the second cell row.

Guanaco (*Auchenia huanaco*).—In general the fibrils are absent, but about the hair follicles, especially, occasional well developed fibrils are seen. One cell in mitosis shows two fibrils embracing the nucleus on either side.

Angora (*Capra ancyrana*).—In this skin numerous fibrils can be seen both in and between the cells of the basement row, some extending into the corium and some extending into the mesial zone of flattened cells. Many of these fibrils are of a distinct corkscrew shape. They lie for the most part at the edge of the cells. Some are in lymph spaces between the cells.

Camel (*Camelus dromedarius*).—This skin was taken from a young animal and shows fair fibre development. The fibrils are seen only in certain parts of the section. The lower cells are usually flat rather than columnar, and certain fibres can be seen running parallel to the corium instead of perpendicular to it as is usually the case.

Axis deer (*Rusa indica*).—In certain areas a few fibrils are seen, rarely extending above the second cell row. The fibrils here lie for the most part at the cell edge.

Llama (*Auchenia llama*).—There is even better development here of the basement fibrils than in the section last described. The fibrils are practically everywhere present but certain areas show especially thick grouping. Some of the fibres are quite broad, and are for the most part straight, or rather,

lightly curved, in conformity with cell contour. They appear most frequently at the edges of the cells.

Zebra (*Equus montanus*) (Fig. 1).—Here the fibres are still more prominent and quite generally distributed. They may be seen both within and at the edges of the basement cells. Some of the fibrils run through the cells of the second row and end in the third row of cells. Very distinct spiral and corkscrew forms may be seen.

Elephant (*Elephas africanus*) (Fig. 2).—The cells of the epidermis are here somewhat crowded together, due apparently to the pressure of the thick, horny layer. The basement fibrils are everywhere present and more strongly developed than in any of the other sections. Where these fibrils approach the cells above there is a suggestion of their unity with the fibrils of this layer which will be discussed later on.

Fibrils appear in the lower cells of the epidermis in certain animals and in general vary in length and thickness. They lie usually at the edge of the cell, appearing at times as a thickened cell membrane. In places they are more or less detached from the cell. They are usually straight or slightly curved, but sometimes assume a distinct spiral form. The lower ends of the fibrils may be in contact with the corium. Sometimes a fibril extends into the corium for a short distance. There is no evidence, however, that these fibrils serve in any way to join the epidermis to the corium. The fibrils are usually limited to the length of the lower row of cells, but sometimes extend well up into the second cell row. In all these animals, excellent pictures are obtained of the fibrils of the stratum filamentosum ("bridges").

I have also found in the skin of Necturus well developed spiral fibres almost completely surrounding the nucleus of each cell of the basement row (Fig. 3). These fibrils are sometimes seen in the cells of the next higher row, but not so well developed. They are situated well in the protoplasm of the cell in the lower row and approach the periphery of the cell when seen in the next row. While there are no "bridges" in the skin of Necturus, these fibres seem to differentiate themselves into a substance that joins together the cells of the upper layers of the epidermis.

Returning to human skin, the fibrils joining together the cells of the stratum filamentosum in conditions apart from normal will first be considered. Increased distinctness of these fibrils

may be seen in human epidermis where the cells are somewhat separated by fluid, as in varying conditions of œdema. In such situations these fibrils may be seen joining together the cells, not only in the stratum filamentosum, but in the stratum granulosum above and the stratum germinativum below. That is, in situations that admit of a proper view of all the cells of the epidermis they are seen to be joined together from top to bottom by delicate fibrils that extend some distance into the cells.

The histologic importance of these fibrils, which are so prominent in both human and animal epidermis, may be emphasized by the fact that I have found them reproduced in skin islets of teratomata exactly as they appear in normal epidermis.

In addition to these fibrils, which are commonly termed the ntercellular bridges, there may be seen in the conditions of œdema above described, especially if the section is taken somewhat distant from the edge of a granulating wound of the skin (Fig. 4) or from the edge of a vesicular lesion such as is found in variola and vaccinia, in addition fine or coarse fibrils, intracellular or in part free from the cell, that are not noticeable in the normal epidermis. These larger fibrils run perpendicularly to the corium and sometimes extend a short distance into it. Some of them are quite long, extending into the second or third row of cells. The end to end juncture of certain of the cells of these rows is seen to be affected by these fibrils. The appearance of these fibrils in this situation and their distribution to the cells above the basement row suggests a unity of the two apparently separate sets of fibrils that have been described.

This suggestion of the unity of these apparently different fibrils above noted, is emphasized in a section taken from the new growing epithelium near the ulcerating edge of an epithelioma of the lip where there is very rapid epithelial proliferation (Fig. 5). This section shows in the stratum germinativum a luxuriant growth of the long protoplasmic fibrils. Not all these fibrils, however, are in the protoplasm. From the rapid pushing up of the epithelium many are freed from the cell, either wholly or in part, and lie in the space between the cells, or lie just at the edge of the cells as an apparent membrane. Some of the

fibrils are quite long, extending upwards a distance of several cells. Occasionally a fibril is seen extending a short distance into the corium. A considerable number of the fibrils have the spiral form that is frequently described. In the stratum filamentosum we find numerous cells joined together by these fibrils. Some are joined end on end but frequently the fibrils deviate and form also a side to side juncture, or again they partially deviate and join the end of one cell to the side of another. Where these fibrils have fully differentiated themselves into the connecting fibrils of the stratum filamentosum they are longer, more irregular, and thicker than the "bridges" in normal epidermis. This is to be expected in newly formed tissue where compensatory growth is always a hypercompensation at first. Moreover, these fibrils do not show the central nodes (Ranvier-Bizzozero nodules) that are prominent later on, and this fact would suggest that the nodes were incident to the shrinkage of the fibres. Everywhere in the section there is the irregularity which is evidence of youth and is comparable to the irregularity of young connective-tissue fibrils. The evenness possessed by structures further removed from adolescence has been attained in no place in the section.

The proof of the unity of these fibrils that is evident in this situation is not confined wholly, however, to situations that show separation of the cells, but may be found under opposite conditions, namely with compression of the cells incident to increased cornification, occurring in association with more or less rapid proliferation of epithelium. Papillomata of the skin (warts) offer material for the further consideration of these fibrils

Under pressure from the increased development of the stratum corneum (Fig. 6) the fibrillar groundwork of the epithelium is very conspicuous. There is only slight separation of the cells and consequent shortening and crowding together of the connecting fibrils. The perpendicular fibrils are very distinct and are best seen in the basement row of epithelial cells where they present a brush-like border to the corium. They are not limited to the lower row or rows of cells, but may also be seen extending into the layer of polygonal cells where they either continue as

perpendicular fibrils or branch off and become the so-called "bridges" of this layer. The unity of the fibre system is everywhere apparent.

Still greater degrees of compression emphasize the fibrillar structure of the epidermis but may be too great to admit a close following of the fibrils. Such a section is seen in epidermis taken from the thickened palm of a laborer (Fig 7) following amputation for extensive phlegmon of the arm. Instead of distinct cells connected by fibrils there is an apparent moulding together of the cell protoplasm into a solid mass. in which the situation of the nuclei. which have more or less completely disappeared, is represented bv round or oval empty spaces. In thin, properly stained sections, this apparently solid protoplasmic mass is seen to possess a fibrillar structure. The fibres are largest and most dense around the nuclear spaces, and it seems reasonable to suppose that the appearance is due to a massing together of the various fibres into what appears to be a deeply staining membrane around the nuclear space such as would naturally be formed when the cells are crowded together. This picture is emphasized by rapidity of growth, and the fibrillar structure is lost except in the basement cells where the edges present a distinct brush-like border to the corium. Not only under the conditions which have just been mentioned is there proof of the unity of fibrils, but in the skin of the elephant previously described, a picture is presented (Fig. 2) suggesting unity of fibrils in the absence both of the separation of cells noted in the first description and the marked compression incident to the processes last described. Here the epidermis is in a state of normal equilibrium. There is not the irregularity incident to rapid proliferation and overgrowth. Consequently, transitional forms of these fibrils are not so numerous as in the more unstable situations. Nevertheless, the unity of the two forms of fibrils is more clearly suggested than in the human epidermis when under normal conditions fibrils are found only in the stratum filamentosum.

The detailed consideration of the relation of these fibrils to malignant growths of epithelial origin has been omitted, as

not enough material was at hand to make a complete study of them. The greatest development of these fibrils, however, was seen in a rapidly growing squamous cell carcinoma of the tonsil. In this tumor there was the type of epithelial proliferation usually associated with this form of growth. Most of the mass consisted of irregular strands and nests of epithelial cells with very little connective-tissue stroma between the cell masses. These cell masses consisted of an outer zone of columnar cells, a mesial zone of polygonal cells, and a centre of flattened (in places cornified) epithelial cells. Throughout the section fibrils could be seen extending through certain cells of the outer zone and extending into and connecting the cells of the mesial zone. These fibrils differed in no way from the fibrils previously described. Arising apparently in the columnar cells, they serve to join together long rows of cells corresponding to those of the stratum filamentosum of normal epidermis. The irregular cell development of the tumor gave bizarre arrangements of fibrils, so that exaggerated reproductions of the process seen in Fig. 5 are apparent in discrete areas throughout the section. This reproduction of fibrils in squamous cell carcinoma, whereby the finer mechanism of epithelial-cell growth can be observed, would leave no doubt of the origin of these tumors if the point should be in question. It seems possible, therefore, that a further study of these fibrils may show them to be of importance in the identification or the grouping of tumors derived from squamous epithelium, since, where growth is most rapid, fibrils are most prominent.

From a general consideration of these specimens it is seen that in all sections of skin certain fibrils are present, which serve to bind together the cells of the epidermis, and are best seen in the stratum filamentosum where the cells are larger and paler and somewhat separated by lymph channels. These fibrils in normal skin are comparatively uniform and arranged with considerable regularity. In conditions of epithelial new growth these fibrils lose their regularity and uniformity, extending in places through several cells and branching off in various directions. Their increased prominence seems to be in direct

ratio to rapidity of cell production. They can be traced in such conditions to fibrils in the stratum germinativum, with which they are in many instances directly continuous. These basement fibrils, which are practically wanting in normal human epidermis, are present in larger numbers in rapidly proliferating epidermis and are seen in many of the lower vertebrates.

Possibly the most important deduction to be drawn from the foregoing study of epidermal fibre production is that it establishes a relationship between the epidermal fibre system and the fibre system of other tissues, namely nervous, muscular, and connective-tissue structures.

We are now familiar with the fibrils of neuroglia cells, thanks especially to the work of Weigert and of Mallory; these cells are embryologically at least related to the cells of the epidermis, since both are of ectodermal origin. It has been shown by Southard[18] that in certain inflammatory processes of the cerebellum there is a change in the neuroglia cells, "such that numerous fibrils are thrown out which are roughly proportional to the number of cells present normally in the reacting area."

Recently, Mallory[19] discovered in connective tissue a hitherto undescribed fibril which he named the fibroglia fibril. These fibrils are produced by connective-tissue cells and are to be found normally in certain situations, but are most abundant in rapidly growing connective tissue, such as granulation tissue and the connective-tissue stroma of rapidly proliferating carcinoma. I have noted that those granulating ulcers of the skin that show the most marked production of epidermal fibrils also show, in the connective tissue below, large numbers of fibroglia fibrils.

It has been shown by Heidenhain[20] that muscle cells possess, in addition to their internal fibrils, certain peripheral fibrils which have been termed myoglia fibrils. These fibrils are, however, remarkably sensitive to post-mortem change, and it is doubtful if their full significance is yet understood. Quite recently fibrils in epithelial cells have been described by Wolbach.[21] He found in a carcinoma of the bladder fibrils varying

in diameter, sometimes one and sometimes several to a cell, running longitudinally in the protoplasm of the cell.

CONCLUSIONS.

In certain animals, in addition to the protoplasmic fibrils of the stratum filamentosum that are present normally in human epidermis, there are well developed fibrils in the cells of the stratum germinativum. These fibrils are present in the human epidermis only under conditions of increased cell activity.

These fibrils, that are prominent in the stratum germinativum of human epidermis under conditions of increased cell activity, seem to increase in direct ratio to rapidity of cell production.

The differentiation of these fibrils of the stratum germinativum into the finer fibrils which connect the cells of the stratum filamentosum can be observed under proper conditions of growth and therefore there should be no separation of the fibrils into different groups with various names.

This process of fibre production by the cells of the human epidermis is analogous to increased fibre production by various other cells under similar conditions, and may be of importance in identifying or classifying new growths of epithelial origin.

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EXPLANATION OF PLATE XXIII.

Fig. 1.—From epidermis of zebra. Well developed fibrils are present both within and at the edges of the basement cells; some are of spiral form. Cells of the second layer are compact and joined by closely set protoplasmic fibrils, the ends of which are lost in the protoplasm of the cells.

Fig. 2.—From epidermis of the elephant. Perpendicular fibrils of the basement cell layer are prominent. The fibrils of the second layer are long, and irregular, reach the nucleus in places, and show distinct Ranvier-Bizzozero nodules.

Fig. 3.—Protoplasmic fibrils in basement cells of Necturus.

Fig. 4.—Œdema of skin with some proliferation of epithelium. A few of the long fibrils appear in the basement cells. Finer fibrils join all the cells (stratum germinativum and stratum spinosum). Ranvier-Bizzozero nodules are prominent.

Fig. 5.—Rapidly growing epidermis adjacent to ulcerating edge of carcinoma of lip. There is rich fibril production and the development of finer fibrils, connecting the cells of the mesial zone, from the long coarse fibrils of the basement cells is apparent. There are no Ranvier-Bizzozero nodules.

Fig. 6.—Papilloma of skin.

Fig. 7.—Palm of laborer showing excessive production of fibres.

Fig. 1

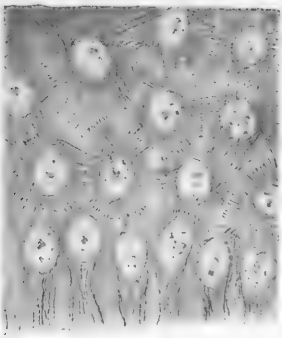

Fig. 2

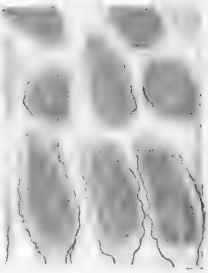

Fig. 3

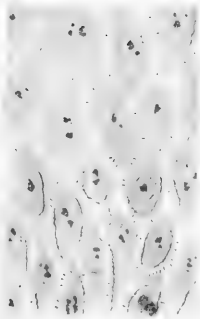

Fig. 4

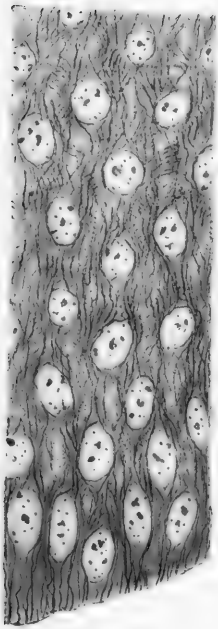

Fig. 6

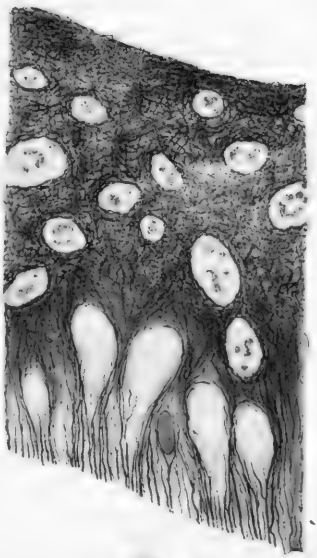

Fig. 7

Fig. 5

ON THE INFLUENCE EXERTED ON THE VIRULENCE OF CARCINOMA IN MICE BY SUBJECTING THE TUMOR MATERIALS TO INCUBATION PREVIOUS TO INOCULATION.

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It is proposed to present in this communication the results of experiments made with a view to influencing the virulence of tumor cells by incubation previous to inoculation. The Jensen mouse carcinoma has been employed exclusively in this series of experiments, and, in view of the extensive literature on the subject, there is no need of further description of the tumor.

Jensen,[1] Loeb, Sticker, and Haaland have all, in the course of their transplantation experiments with animal tumors, determined the point at which the tumor materials, through exposure to high temperature, previous to inoculation, were rendered incapable of further proliferation. Jensen, working with his mouse carcinoma, found that exposure of the tumor materials suspended in normal salt solution (0.8 per cent.) to temperatures of 47° C. and upwards for a period of five minutes entirely destroyed the tumor cells, whilst at a temperature of 46° C. a distinct dimin tion in the yield of tumors, as compared with that obtained at lower temperatures, was observed. The number of animals employed in this experiment was extremely small, no control was made at normal temperatures, and no statements were made regarding the relative speed at which individual tumors in the different series developed.

Loeb[2] observed that exposure of the rat sarcoma, with which he was working, to temperatures of 45° C. and upwards for periods of thirty minutes or more, was sufficient to prevent

[1] Jensen, *Centralblatt f. Bakteriologie*, Abt. I, Orig., 1903, xxxiv, 122.

[2] Loeb, *Virchow's Archiv*, 1903, clxxii, 345.

further development on transplantation. In one case, employing materials which had been incubated forty minutes at 43° C., a fairly good yield was obtained (four fatal tumors in seven rats). He found, however, in those cases in which temperatures approaching 43° and 44° C. had been used, that the tumors made their appearance later than in the control series and that their speed of development was markedly retarded.

Sticker,[3] in an extensive series of experiments on the transplantation of a lymphosarcoma in dogs, made a single experiment in which small pieces of the tumor were subjected to a temperature of 50° C. for two hours previous to their introduction subcutaneously into a two-months-old puppy. Unfortunately the dog died of pneumonia ten days after the commencement of the experiment, but examination showed tumors to be developing at both points of inoculation, indicating that lymphosarcoma of this type was capable of resisting much higher temperatures than were the materials employed in the experiments recorded above.

Haaland,[4] in a recent communication from Ehrlich's laboratory, published the results of a series of experiments carried out on a mixed mouse tumor with a view to obtaining a sarcoma free from carcinoma cells. The tumor materials were introduced into thin-walled capillary tubes which were heated in a water-bath at a temperature of 44° C., for periods of five, ten, and fifteen, up to sixty-five minutes respectively. In a control series of six mice which had received normal materials without previous incubation, 80 per cent. yielded rapidly-growing tumors, whilst in the heated series the yield of tumors and the speed of development both diminished with the increasing period for which the materials had been incubated previous to inoculation. It was also observed that the carcinoma cells succumbed earlier than the sarcoma cells, with the result that materials which had been treated for thirty-five minutes and upwards yielded pure sarcomata in which no trace of carcinoma was to be found. Haaland considered that the reduction in virulence and speed of

[3] Sticker, *Zeitschrift f. Krebsforschung*, 1904, i, 413.
[4] Haaland, *Berl. klin. Wochenschr.*, 1906, xliii, 40.

development was not attributable solely to the destruction of a large proportion of the cells, but rather to some deep-seated change in the cell mechanism, indicated by the histological findings.

It will be observed that all these authors made use of relatively high temperatures and for the most part of fairly virulent materials, confining their attention principally to a determination of the point at which the cells employed were no longer capable of proliferation. From the statements of Loeb and Haaland, it is apparent that not only was the number of tumors in parallel series decreased with increasing temperatures to which the materials had been exposed previous to inoculation, but, further, a distinct retardation in the development of individual tumors of the high-temperature series was noted by both observers.

All those who have carried on extensive experiments on the transplantation of animal tumors are aware of the extraordinary variations which occur in one and the same series, both in the percentage of tumors and in the speed of development of individual tumors of the series, all of which might be attributable to a variation in the concentration of a stimulating agent causing proliferation of the cancer cells.

Our experiments started with the conception that exposure to temperatures slightly above the normal for short periods of time might exert an effect upon weak and attenuated tumor cells, stimulating them to more active development on subsequent inoculation in a manner analogous to the action of poisons at high dilution on yeasts, bacteria, etc. The activity of catalytic chemical agents on enzymes, toxins, etc., has been demonstrated to increase with increasing temperatures, and since the stimulating effect of poisons is exerted only within very narrow limits of concentration, it is quite conceivable that a slight rise in temperature might so increase the activity of a stimulating body already present as to lead to a marked increase in the proliferative energy of the cell.

Before commencing the series of experiments in which it was necessary to make relatively accurate comparisons between the

results obtained in experimental series and their controls, it was necessary to eliminate as far as possible all sources of experimental error calculated to introduce accidental variations. Our general procedure when inoculating mice, both in these and other experimental series, was as follows: a mouse having a suitable tumor was killed, the tumor removed and weighed and ground in a sterile mortar with three or four times its volume of salt solution. All coarse particles which might interfere with the uniformity of the mixture were removed by means of rakes and in some cases by filtration through coarse gauze, a procedure which, whilst calculated to afford a perfectly homogeneous mixture for experimental work, would also tend to reduce the percentage of tumors. The contents of the mortar were then measured and divided into three or four equal parts in sterilized test tubes. One portion was reserved for purposes of control, the remaining tubes being subjected to the required temperatures for a definite period of time in the Ostwald water-bath. After the completion of the incubation process, the contents of each test tube were introduced into a fresh sterile mortar, once more thoroughly admixed, and uniform doses, usually from 0.4 to 0.5 cubic centimeter of the mixture injected subcutaneously into each mouse of the series. The greatest care was observed in carrying out the series of experiments that the mice selected were all derived from the same source and approximately the same age, so that, employing for example, ten mice for the normal materials and ten each for those which had been incubated at each temperature, it might be said that the only varying factor introduced into these experiments was the influence exerted on the cancer material by subjecting it to incubation previous to inoculation.[5]

[5] As a control on the incubation experiments in this paper, the results obtained by inoculating two or more lots of mice on the same day with the same normal materials are of value. Without observing as extensive precautions as in the experimental series, the yields obtained in various comparable groups were so nearly uniform as to make it extremely probable that the results observed in the incubated series were attributable to the experimental treatment and not to accidental distribution. For example, lots 7 α and 7 β, inoculated June 10, 1904, with the same material, yielded, respectively, 4 tumors in 13 mice and 4 tumors

The mere enumeration of the tumors obtained would of course afford but a very inadequate idea of the relative virulence of tumor materials employed for the inoculation of comparative series. We were led, therefore, early in the course of our experimental work to adopt a system of identifying individual members of a group and following the development of their tumors at short intervals of time from inoculation to death. For this purpose chart books were employed in which the groups were arranged numerically and the individual members of a group alphabetically. For each individual mouse a series of diagrams was prepared showing the fore legs, hind legs, nose, and tail. When a tumor made its appearance, it was reproduced in the location in which it occurred, in a diagram as nearly life size as possible, and as the tumor increased in bulk and secondary tumors made their appearance, further chartings were prepared showing the progress of the growth until the death of the animal occurred or spontaneous recovery intervened. (Gaylord and Clowes, On Spontaneous Cure of Cancer, *Surgery, Gynæcology, and Obstetrics*, 1906, ii, 633.)

Making use of such a system, it was possible to determine not only the actual number of fatal tumors in each group, but also the relative speed of development of individual tumors in the series, as indicated by the number of days from inoculation required to reach a certain size, and the volume of the tumors after a given number of days.

It would of course be preferable in a publication of this nature to include the complete charted records of every series of experiments, but since, on account of space limitations, this is impossible, reference has been made to the records wherever necessary in order to bring out the essential points.

The greatest variation in the virulence of tumors is frequently observed in the same series. The age of a tumor, that is to say,

in 11 mice. Lots 161 and 162 each of ten mice, inoculated Dec. 21, gave 3 large tumors apiece. In other cases a difference of one or at most two tumors is observed, but in no normal cases are the differences in the yields as great as those recorded in the incubated series dealt with subsequently in this paper, in spite of the fact that practically no precautions were observed to insure uniformity.

the period which has elapsed between the date of inoculation and the date on which the tumor has attained a size suitable for transplantation, frequently bears a marked relation to the yield of tumors obtained in the following generation. An illustration of this form of variation is to be found in the following table in which are compared the yields obtained on further transplantation from a series of six tumors, all of which belonged to the same group, having been inoculated on the same day with the same materials.

Tumor	Age in days	No. mice employed	No. of tumors	Percentage of tumors.
214 A	45	19	8	42.0
216 B	56	7	2	28.5
213 B	59	38	11	29.0
214 B	66	36	10	28.0
Total 66 days and under		100	31	31.0
218 E	184	26	5	19.2
215 B	184	32	2	6.0
Total 184 days		58	7	12.0

It will be seen that the yield has fallen from 31 per cent. in the rapidly growing tumors to 12 per cent. in those which required upwards of six months for development.

It has also been noted that very young tumors weighing less than four grams seldom give good results on transplantation. In fact, the best yields are obtained, as a rule, from tumors that are still developing fairly actively and weigh over five grams.

After preliminary experiments from which it was concluded, that no tumors could be obtained from materials incubated at temperatures above 44° C. for thirty minutes or more, we commenced the following series of experiments.

Experiment I.[6] November 19, 1904.

[6] In order to avoid confusion, we have tabulated the results as far as possible. Under the lot numbers of comparable experimental series are found first the number of mice employed, followed by the treatment to which the tumor materials had been submitted previous to inoculation, and the results of examination of such series after a period of 8 months.

Mouse tumor 105 C employed for inoculation. Weight of mouse, 18.5 grams. Weight of tumor, 6.5 grams.

Age of tumor (time elapsed from date of inoculation to Nov. 19), 40 days.

Ratio of tumor to salt solution, 1:4. Dose, 0.5 c.c.

Serial No.	Lot 142	Lot 143	Lot 144
Number of mice	8	8	8
Incubation of tumor materials previous to inoculation	Nil	30 mins. at 40°C.	30 mins. at 45°C.
Examination after 3 mos.	6 large tumors	1 large tumor	Nil

In the above series of experiments the tumor employed for inoculation was derived from an extremely virulent strain and incubation at 40° C. was found to lower the yield of tumors from 75 to 12.5 per cent.[7]

Experiment II. December 5, 1904.

Mouse tumor 101 D employed. Weight of mouse 22.5 grams. Weight of tumor, 3.5 grams.

Age of tumor, inoculation to death, 66 days.

First appearance of tumor to death, 28 days.

Ratio of tumor to salt solution, 1:4. Dose, 0.5 c.c.

Serial No.	Lot 119	Lot 150	Lot 151
Number of mice	8	8	8
Incubation of tum. mat. previous to inoculation	Nil	10 min. at 35° C.	10 min. at 40° C.
Examination after 3 mos.	4 large tumors	2 tumors 1 spon. recovery	1 large tumor

The tumor employed in this experiment was also derived from a virulent strain and although small it developed rapidly in the course of the few days preceding date on which the animal was killed. It was moderately necrotic and from its appearance was expected to yield a fair proportion of tumors. As has been seen from the table, the yield decreased with increasing temperatures to which the materials were submitted previous to inoculation.

[7] The percentage terms used in this case are simply for purposes of comparison, as it would obviously be impossible to make any calculations based upon such a small number of animals.

Experiment III. March 17, 1905.

Mouse tumor 251 E employed. Origin, 142–105.[8] Weight of mouse, 52.5 grams. Weight of tumor 6.2 grams.

Age of tumor, inoculation to death, 53 days.

From first appearance of tumor to death, 36 days.

Ratio of tumor to salt solution, 1:4. Dose, 0.5 c.c.

Serial No.	Lot 166	Lot 174	Lot 190
Number of mice	9	9	6
Incub. of tum. mat. previous to inoculation	Nil	15 min. at 40° C.	15 min. at 45° C.
Period after inoculation.	Record of results.		
10 days.	2 small tum.	6 small tum.	Nil
15 days.	2 of 5 mm. diam. 2 of 3 mm. diam.	3 of 10 mm. diam. 3 of 6 mm. diam.	Nil
31 days.	2 of 10 mm. diam. 2 of 5 mm. diam.	1 death of tum. 4 lg. 1 sm. tum.	Nil
Final results, 4 months.	3 large tum. 1 small tum.	5 large tum. 1 small tum.	Nil

As a final result it will be seen that five large tumors and one small tumor occurred in the incubated series in comparison with three large and one small in the normal series. In addition, it should be noted that the speed of development of the tumors in Lot 174 was far greater than that of the tumors of Lot 166. Fifteen days after inoculation the former were as far advanced as were the latter at the end of 31 days. Lot 174 afforded us a remarkable illustration of extremely rapidly developing tumors which became necrotic and were suitable for transplantation at a much earlier date than was normally found to be the case.

Experiment IV. April 7, 1905.

Mouse tumor 198 B employed. Origin 150 B–101. Weight of mouse 27.4 grams. Weight of tumor, 6.6 grams.

Age of tumor, inoculation to death, 38 days.

First appearance of tumor to death, 27 days.

Ratio of tumor to salt solution, 1:3.

Materials filtered through gauze and consequently considerably attenuated. Dose, 0.4 c.c.

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[8] In this and subsequent cases in which the origin of the tumor is given its direct descent is traced to one of the four groups, 101, 102, 105, 106, all of which were in the second generation from the original Jensen tumor. For family tree showing relationship of individual groups see *American Journal of Physiology*, 1905, xiv, 177.

Serial No.	Lot 227	Lot 224	Lot 225	Lot 226
Number of mice	6	6	6	6
Incubation	Nil	30 min. at 40° C.	2 hr. at 40° C.	19 hr. at 40° C.
Examination after 4 Mos.	No tumors	1 tumor	1 tumor	No tumors

The low yield of tumors in this series is probably attributable in part to the filtration through gauze but it should also be noted that the tumor in question was young and in active development and showed very limited areas of necrosis.

Experiment V. April 19, 1905.

Mouse tumor 41 B employed. Origin of tumor, 147–105. Weight of mouse 30.5 grams. Weight of tumor, 5.2 grams.

Age of tumor, inoculation to death, 62 days.

First appearance of tumor to death, 40 days.

Ratio of tumor to salt solution, 1:4.

Material filtered through gauze. Dose, 0.4 c.c.

Serial No.	Lot 234	Lot 235
Number of mice	6	6
Incubation	Nil	1 hour at 41° C.
Examination after 6 mos.	1 slow-growing tumor weighing 1 to 2 grams	4 large rapidly growing tumors weighing 5 to 9 grams.

Experiment VI. April 19, 1905.

Mouse tumor 199 F employed. Origin, 150 B–101. Weight of mouse, 24.2 grams. Weight of tumor, 9.3 grams.

Age of tumor, inoculation to death, 50 days.

First appearance of tumor to death, 38 days.

Ratio of tumor to salt solution, 1:4.

Material filtered through gauze. Dose, 0.4 c.c.

Serial No.	Lot 236	Lot 237
Number of mice	7	6
Incubation	Nil	1 hour at 41° C.
Examination after 4 mos.	1 slow-growing tumor	1 rapidly growing tumor.

The extreme attenuation in this group resulting in the production of one tumor only in each of the series is probably attributable to the filtration through gauze.

The four preceding series are remarkable in that in no case is there found to be a smaller yield of tumors in the groups incubated at 40° or 41° C. than in those which received no treatment. Summarizing the results it may be said that whilst six slow-growing tumors were obtained from thirty mice in the normal series, ten rapidly growing and two slow-growing tumors, making

a total of twelve tumors, were obtained from twenty-nine mice in the groups incubated at 40° or 41° C. If we exclude the two series in which filtration through gauze has led to such a marked attenuation that four tumors only were obtained from the inoculation of thirty-nine mice, we find that in the two remaining series five slow-growing tumors were obtained from fifteen mice in the normal groups, as against eight fast- and one slow-growing tumors out of fifteen mice in those groups which had received injections of tumor materials previously incubated at 40° or 41° C.

Having thus demonstrated that incubation at relatively high temperatures is not necessarily prejudicial, and, in the case of those tumors which afforded a small yield at normal temperatures, actually led to an increase, we determined to employ tumors known to possess a high degree of virulence. For this purpose two tumors of Lot 174 were chosen for the following experiments, VII and VIII. This group, referred to above in Experiment III, was characterized by the extreme rapidity with which the tumors of its members reached maturity, as indicated by a peculiar type of non-infected necrosis, which has been found on experiment to yield the maximum of tumors on transplantation.

Experiment VII. May 5, 1905.

Mouse tumor 174 G employed. Origin, 251 E–142–105. Weight of mouse, 22.0 grams. Weight of tumor, 5.2 grams.

Age of tumor, inoculation to death, 49 days.

First appearance of tumor to death, 35 days.

Tumor partially necrotic but not infected.

Ratio of tumor to salt solution, 1:4. Dose, 0.5 c.c.

Serial No.	321	322	323	324
Number of mice	8	8	8	8
Incub. of tum. mat. previous to inoc.	Nil	1 hr. at 40° C.	1 hr. at 42°C.	1 hr. at 44°C.
Period after inoculation		Record of results		
24 days.	4 large tum. 2 small tum.	1 large tum. 3 small tum.	Nil	1 small tum.
Final results after 4 mos.	6 large tum. 1 intermed. t.	3 large tum. 1 small tum.	1 small tum.	1 large tum.

It will thus be seen, to summarize the results obtained by making use of this extremely virulent material, that employing eight mice in each case, seven tumors were obtained in the normal series, four in the series incubated at 40° C., and one each in the series incubated at 42° and 44° C.

Experiment VIII. May 16, 1905.
Mouse tumor 174 B employed. Origin, 251 E–142–105. Weight of mouse 24.8 grams. Weight of tumor, 4.3 grams.
Age of tumor, inoculation to death, 60 days.
First appearance of tumor to death, 50 days.
Tumor partially necrotic but not infected.
Ratio of tumor to salt solution, 1 : 4.5. Dose, 0.5 c.c.

Serial No.	329	330	331	332
Number of mice	8	8	8	8
Incub. of tum. mat. previous to inoc.	Nil	1 hr. at 39° C.	1 hr. at 41° C.	1 hr. at 43° C.
Period after inoc.		Record of Results		
28 days	1 large, 2 mod. and 1 small tum.	1 mod. tumor	1 mod. tumor	Nil
Final results, 4 months	5 large tumors	3 large tumors	2 large tumors	Nil

To summarize the results it may be said that there developed in the normal series five tumors, in the series incubated at 39° C. three tumors, in that at 41° C. two tumors, and at 43° C. nil.

In the two preceding series, VII and VIII, we were dealing, as in Experiments I and II, with materials above the normal average of virulence and in both cases incubation appears to diminish the proportion of successful transplantations, and, further, to effect a slight retardation in the speed of development as compared with the normal.

On May 19 and 23, two series of experiments were carried out in which the materials were incubated two hours at 43° to 44° C. previous to inoculation. This treatment produced such a marked inhibition, only one tumor being obtained in the entire series, that we have excluded the experiment in question and must assume that such a prolonged treatment at a temperature as high as 44° C. is capable of entirely destroying the tumor cells, thus rendering further proliferation impossible.

An analysis of the results obtained in the preceding eight series made it appear probable that whilst treatment at a temperature of 38° to 41° C. was distinctly unfavorable to the development of virulent tumors, the same process produced a stimulating effect leading to an increased proportion of tumors as compared with the normal when non-virulent materials were employed.

In the six remaining series an attempt was made to compare the results of slowly and rapidly growing tumors, of small and large tumors, etc.

Experiment IX. October 11, 1905.

Mouse tumor 340 A employed. Origin, 213 B–175–161–128–101. Weight of mouse, 32.75 grams. Weight of tumor, 6.4 grams.

Age of tumor, inoculation to death, 138 days.

First appearance of tumor to death, 119 days.

Tumor only slightly necrotic.

Ratio of tumor to salt solution, 1:3. Dose, 0.6 c.c.

Serial No.	469	470	471
Number of mice	8	8	8
Incub. of tum. mat. previous to inoc.	Nil	30 min. at 38.5° C.	30 min. at 42.2° C.
Period after inoc.		Record of results	
41 days	1 mod. tumor 1 small tumor	2 mod. tumors 2 small tumors	2 small tumors
58 days	1 mod. tumor 1 small tumor	2 large tumors 2 small tumors	1 small tumor
Final results, 4½ months	2 mod. tumors	2 large tumors 1 small tumor	Nil

Experiment X. October 11, 1905.

Mouse tumor 427 A employed. Origin, 395 C-232 B-195 D-169 C-106. Weight of mouse, 37.9 grams. Weight of tumor, 9.9 grams.

Age of tumor, inoculation to death, 43 days.

First appearance of tumor to death, 30 days.

Tumor far more necrotic than 340 A used in Experiment IX.

Ratio of tumor to salt solution, 1:3. Dose, 0.7 c.c.

Serial No.	472	473	474
Number of mice	10	10	10
Incub. of tum. mat. previous to inoc.	Nil	30 min. at 38.5° C.	30 min. at 42.2° C.
Period after inoc.		Record of results	
41 days	4 mod. tumors 2 small tumors	2 mod. tumors 3 small tumors	Nil
58 days	4 large tumors 2 mod. tumors	1 large tumor 1 mod. tumor	Nil
Final Results, 4½ months	6 large tumors	1 large tumor 1 mod. tumor	Nil

The above results obtained in Experiments IX and X are interesting in view of the fact that they afford us a comparison between two tumors which showed a great difference in their speed of development. 340 A, used in Experiment IX, was inoculated on May 26, showed a tumor as early as June 14, which increased in size until June 26, on which date it diminished and remained stationary for a couple of months, finally

about the beginning of September developing once more until on October 11 it had reached a sufficient size to be used for transplantation.

Mouse No. 427 A, used in Experiment X, was inoculated August 29 and its tumor had reached considerable dimensions by September 18 and as stated above weighed almost 10 grams on October 11. Thus the tumor which required 138 days to reach 6.4 grams showed practically uniform results in the three series of experiments, an actual increase in the number of tumors being noted at a temperature of 38.5° C., whilst the tumor which required only forty-three days to reach a weight of 9.9 grams and showed marked necrosis, gave a large yield, 66 per cent., at normal temperatures, falling to 22 per cent. at 38.5° C., and to nothing at 42° C.

Experience had shown that small, hard tumors gave extremely poor yields on transplantation. We determined therefore to make use of two tumors, both of which had been inoculated on the same day, one of which had grown slowly and the other rapidly.

Experiment XI. October 25, 1904.

Mouse tumor 429 A employed. Origin, 328 C–214 A–175 C–161 M–128–101. Weight of mouse, 24.7 grams. Weight of tumor, 4.2 grams.

Age of tumor, inoculation to death, 48 days.

First appearance to death, 30 days.

Tumor small, hard, and not necrotic.

Ratio of tumor to salt solution, 1:3. Dose, 0.5 c.c.

Serial No.	505	506	507
Number of mice	10	10	10
Incub. of tum. mat. previous to inoc.	Nil	20 min. at 36° C.	20 min. at 40.5° C.
Period after inoc.		Record of results	
27 days	3 small tumors	2 mod. tumors 4 small tumors	1 large, 1 mod., and 5 small tum.
44 days	2 small tumors	3 large tumors 3 med. tumors	4 large, 1 mod., and 2 small tum.
Final results, 4 months	1 small tumor	5 large tumors 1 med. tumor	4 large tumors 1 med. tumor

Experiment XII. October 25, 1904.

Mouse tumor 432 B employed. Origin, 329 E–174 B–100 E–142–105. Weight of mouse, 36.2 grams. Weight of tumor, 11.8 grams.

Age of tumor, inoculation to death, 48 days.

First appearance of tumor to death, 25 days.

Tumor large, soft, cystic, and necrotic but not infected.
Ratio of tumor to salt solution, 1:3. Dose, 0.5 c.c.

Serial No.	508	509	510
Number of mice	10	10	10
Incub. of tum. mat. previous to inoc.	Nil	20 min. at 36° C.	20 min. at 40.5° C.
Period after inoc.		Record of results	
27 days	2 med. tumors 1 small tumor	1 med. tumor 2 small tumors	1 med. tumor 1 small tumor
44 days	2 large tumors 2 med. tumors	1 large tumor 3 med. tumors	1 large tumor
Final results after 4 months	2 large tumors 2 med. tumors	4 large tumors	1 large tumor

Comparing the results of the two preceding experiments it will be seen that whilst the incubation of the small, hard tumor led to a great increase, from 10 to 60 per cent., in the yield of tumors in the succeeding generation, the incubation of the large, soft, cystic tumor produced relatively less effect, a decrease in the proportion of tumors at 40° C. being noted, as compared with the normal.

The final series of incubation experiments was carried out making use of two slow-growing tumors both of which had shown at some stage more or less tendency to remain stationary or retrogress.

Experiment XIII. December 12, 1904.
Mouse tumor 419 B employed. Origin, 383 A–304 A–200 A–150 B–101.
Weight of mouse, 18.8 grams. Weight of tumor, 2.9 grams.
Age of tumor, inoculation to death, 112 days.
First appearance of tumor to death, 80 days.
Ratio of tumor to salt solution, 1:4. Dose, 0.4 c.c.

Serial No.	590	591	592
Number of mice	8	8	8
Incub. of tum. mat. previous to inoc.	Nil	30 min. at 37° C.	30 min. at 40° C
Period after inoc.		Record of results	
Final results after 10 weeks	2 large tumors	4 large tumors 1 mod. tumor	1 large tumor 1 small tumor

Experiment XIV. December 12, 1904.
Mouse tumor 462 C employed. Origin, 218 E–175 C–161 M–128–101.
Weight of mouse, 33.6 grams. Weight of tumor, 5.3 grams.
Age of tumor, inoculation to death, 74 days.
First appearance of tumor to death, 40 days.
Ratio of tumor to salt solution, 1:3. Dose, 0.5 c.c.

Serial No.	593	594	595
Number of mice	10	9	10
Incub. of tum. mat. previous to inoc.	Nil	30 min. at 37° C.	30 min. at 40° C.
Period after inoc.		Record of results	
Final results after 10 weeks	2 large tumors	Nil	4 large tumors

These two series show, as would be expected, a rather larger proportion of tumors in the incubated than in the normal series; whilst 419 B shows a maximum at 37° C., 462 C gives the highest yield from materials incubated at 40° C. The complete absence of tumors in lot 594 is extremely difficult to explain, and forms the most serious exception in the entire series of experiments enumerated above. We have endeavored to find some explanation for this discrepancy. The ulceration of tumor materials in some mice of this series would indicate a possible contamination during the course of the incubation process. We are not however aware of any such accident having occurred, and must therefore conclude that in spite of the utmost precautions, discrepancies will occur when dealing with materials of this nature.

Excluding the two series of experiments in which filtration through gauze or exposure to temperatures above 44° C. for more than an hour would materially interfere with results, we find that twelve series of experiments have been carried out employing thirty-seven lots of mice, each of which contained from six to ten individuals.

We have included two tables in which the results obtained after three months by incubating five strong and five weak strains at 40° to 41° C. are collected. In the first column is given the number of the mouse employed originally for inoculation of the series. In the second column is the number of mice employed in each series both for the normal and incubated material. In the third column is found the number of tumors present in the normal series, the number preceding the + sign representing the rapidly growing tumors and that following, the slowly growing tumors. In the final column is found the number of rapidly and slowly growing tumors in the incubated series.

TABLE I. VIRULENT GROUPS.

Mouse	Number of mice in each series	Tumors of normal series	Tumors of incubated series
105 C	8	6 + 0	1 + 0
101 D	8	4 + 0	1 + 0
174 G	8	6 + 1	3 + 1
174 B	8	4 + 1	1 + 1
432 B	8	2 + 2	1 + 0
Total	40	22 + 4	7 + 2
		55% + 10% = 65%	17.5% + 5% = 22.5 %

TABLE II. NON-VIRULENT GROUPS.

251	9	3 + 1	5 + 1
41 B	6	0 + 1	4 + 0
429 A	7	0 + 1	4 + 1
419 B[9]	8	2 + 0	1 + 1
462 C	10	2 + 0	4 + 0
Total	40	7 + 3	18 + 3
		17.5% + 7.5% = 25%	45% + 7.5% = 52.5%

In some cases the maximum is obtained at 37° or 38° C., a fall already being noticed at 40° and 41° C. Temperatures of 42° C. and upwards seem in all cases to exert an inhibiting effect upon the tumor development.

An inspection of the above table shows that by incubating the tumor materials in salt solution for varying periods of time at a temperature equal to the maximum fever temperature, a greatly increased yield was obtained from relatively weak strains, the total number of tumors being more than twice as great in the same number of mice. On the other hand, incubation at the same temperature produced a marked inhibiting effect where highly virulent materials were employed, a fall to less than one-third of the number of rapidly growing tumors and from 65 to 22.5 per cent. in the total yield being recorded.

Finally, reference should be made to practical application of incubation as a means of stimulating proliferation of tumors. The transplantation of a primary mouse carcinoma presents far greater difficulties than does the reinoculation of such a tumor in subsequent generations. Any means, therefore, of stimulating the cancer cells to more active proliferation must possess con-

[9] 419 B gave a maximum yield of 50 % on incubation at 37° C. and presumably was already suffering attenuation when heated to 41° C.

siderable value from an experimental standpoint. Several mice having primary carcinoma similar to that of the Jensen mouse have been sent to the laboratory from a common source in the course of a few months. All attempts which were made to transplant the carcinomatous tissue by ordinary means having failed, it was decided to make an attempt to increase the virulence of any subsequent tumor of this group by incubation previous to inoculation. This method afforded extremely satisfactory results, a considerable tumor being obtained in a mouse inoculated with materials which had previously been incubated twenty minutes at 39° C. This tumor was incubated and transplanted, and three tumors were obtained in the following generation. The tumor has now been transplanted through three generations and may therefore be looked upon as successfully established.

The most remarkable facts in connection with this experiment are:

1. Whilst 27 mice which have received incubated material have yielded 8 tumors, 15 mice which received identically similar materials, but without incubation previous to inoculation, have yielded no tumors.

2. The virulence of the tumor has increased in the course of three generations to a marked degree. Whilst ten weeks were required for the development of the first tumor, and five weeks for those in the second generation, tumors of considerable size have already made their appearance in the third generation in less than a week.

We do not attribute any special significance to a single experiment of this nature, but since it appears probable that incubation was instrumental in establishing this series, we are making use of incubation for half an hour at temperatures of 38.5° to 39.5° C. in all cases in which primary tumors are sent to the laboratory, and have included a note on the subject in the hope that our method may possibly prove of value to others working in the same field.

SUMMARY AND CONCLUSIONS.

Our experience with mouse tumors has demonstrated that a great difference exists not only in the relative immunity possessed by different individuals but also in the relative virulence of different tumors all owing their origin to the same source.

In this paper it has been shown that the variations in question

can be accentuated by incubation of the transplantation materials previous to inoculation at temperatures varying from 37° to 41° C. In the case of extremely virulent tumors giving a yield of upwards of 60 per cent. under normal conditions, a distinct and gradual fall in virulence accompanies increasing temperatures of incubation until a point is reached at which no further tumors are obtained. On the other hand, relatively inactive materials giving comparatively small yields of tumors under normal conditions may be so stimulated by subjecting them to incubation at suitable temperatures as to afford a larger yield and relatively more rapidly-growing tumors than were obtained in the normal series. In these cases the optimum temperature (that is to say, the temperature at which, when incubated for a definite period of time the materials employed yield the largest proportion of tumors) appears to vary, for whilst the most virulent materials, as stated above, exhibit an optimum yield at normal temperatures decreasing with incubation, less virulent materials show an optimum at temperatures slightly above the normal, whilst the least virulent of all show an optimum at as high a temperature as 40° to 41° C., all the curves eventually falling to zero, as those temperatures are reached at which the cells themselves might be expected to succumb.

This fact having been demonstrated, it remains to compare the histories of the various tumors employed for the sixteen series of experiments. The charted diagrams representing the course of development of each individual tumor from the date of inoculation to the date of death have been prepared and afford an admirable means of comparison of the relative speed of development of the tumors employed and consequently of their relative proliferative energy. The origin of all tumors for several generations, whether from a relatively active or inactive stock, may be determined from the records, and data regarding the age, weight, and pathological state of the tumor are also available.

1. Those tumors which give a small yield under normal conditions, but which give an increased yield on incubation, are either rapidly growing, young tumors, or small and hard tumors, or have been extremely slow in their development, and have in

certain cases exhibited indications of a tendency to undergo spontaneous recovery. Tumors of this class seldom exhibit any marked indications of necrosis of the type which will be referred to in the next paragraph.

2. Those tumors which on inoculation give a large yield at normal temperatures and a gradually decreasing percentage of tumors in the batches inoculated with materials which have been incubated at increasing temperatures are generally fully grown tumors which have developed rapidly and come of a relatively active strain. They are usually softer and more cystic than those considered in the previous section. Their most marked characteristic is, however, a state of necrosis accompanied by an increasing calcium content without an entire disappearance of potassium.[10] This state of necrosis is peculiar in that it is apparently not attributable to the intervention of bacteria, very few of which are present in the most satisfactory transplantation materials, but rather to some auto-digestive or enzymotic process at work within the cells.[11]

The enormous proliferative power possessed by the cells of this Jensen tumor has frequently been referred to by other workers who have employed the same materials. Such a remarkable development may most readily be explained as due to the intervention of some agent continually or intermittently stimulating the cells to further proliferation. We are not concerned at the present moment with the exact chemical composition of the body in question, as to whether it is possessed of an enzymotic or toxic nature or consists simply of a complex aggregate possessing a well-defined chemical composition. Neither are we attempting to determine the source of the body in question; whether it be derived from some parasite which has invaded the cell, or is itself a product of the impaired metabolism of the cancer cell. It is simply proposed to consider the probable action,

[10] Clowes and Frisbie, *American Journal of Physiology*, 1905, xiv, 173.

[11] In all those cases in which tumors have been infected they have proved totally unfit for transplantation experiments, leading either to the death of the mouse or to an ulceration process whereby the infected materials were entirely removed, no tumors resulting from such a treatment.

at varying concentrations, of a chemical body capable of exerting a stimulating action on the cell which reaches an optimum at a given concentration and follows certain laws governing chemical action.

Poisons which cause the death of an organism at concentrations exceeding a given limit are in all cases capable of producing a stimulating effect upon that same organism when employed at sufficient dilution, whilst below that optimum point they produce little or no effect of any kind. Thus, for example, mercuric chloride inhibits absolutely the development of yeast at a concentration of one part in 20,000, whilst at a dilution of 1 in 330,000 the proliferative power of the yeast subjected to its action is actually increased.[12] The same effect is exerted upon enzymes; diastase, for example, is stimulated in its activity by the addition of extremely small amounts of sulphurous acid, oxalic acid, etc., whilst had the concentration been ten to forty times as great, the action of the diastase would have been nil, or practically inhibited.[13] It should be noted under this head that the stimulating effect of such toxic bodies is exerted only within extremely narrow limits, usually ranging from concentrations about five times as great to concentrations about five times as dilute as the optimum.

A second law which is applicable to the case in point is that governing the action of chemical bodies in solution at the same concentration but at varying temperatures, to the effect that with rising temperatures their activity is correspondingly increased. This law has been found to apply to bodies of the nature of enzymes, toxins, etc. Thus, for example, the activity of invertase in the conversion of cane-sugar into invert-sugar increases with increasing temperature until a point is reached at which physical conditions, such as precipitation of the enzyme, lead to the suspension of its function and to a decrease in activity.

The recent work of Arrhenius and Madsen[14] on the hæmolysis of red blood corpuscles, making use of dilute solutions of toxins,

[12] Biernacki, *Pflüger's Archiv*, 1891, xlix, 112.
[13] See Duclaux, Traité de Microbiologie, vol. ii.
[14] Festskrift af Indvielsen ved Statens Seruminstitut, Copenhagen, 1902.

such as diphtheria and tetanus, has demonstrated that these bodies exert a hydrolytic effect very similar to that of weak acids and bases and following the physico-chemical laws governing dilute solutions both as regards concentration and temperature variations.

If, as is highly probable, the proliferation of tumor cells is attributable to the presence of a toxic or chemical body acting as a stimulus, the concentration of the body in question most favorable to active proliferation probably lies within comparatively narrow limits, and consequently the slight changes in the concentration of this body would produce a marked variation in stimulus.

The second law considered above regarding the influence of temperature makes it appear probable that increased temperatures at a given concentration would produce a result similar to increase in concentration at the same temperature; in other words, an increase in stimulus to proliferation. Thus, where the concentration is too low to produce a marked effect at normal temperatures, an increased activity may be brought about by increased temperatures, the chemical energy of the stimulating body being thereby increased. On the other hand, the incubation of materials in which the stimulant was already present in the most favorable proportions for the normal temperatures would so increase the activity of the body in question as to exceed the optimum and eventually exert a destructive, inhibiting action upon the development of the cells themselves.

That so short a treatment in saline solutions at temperatures equal to or not far in excess of the normal should produce so marked an effect may at first sight appear impossible, but the effect of the highly dissociated saline solution employed must not be ignored, and for rapidity of effect we have a perfect analogy in the results published by Calkins[15] on the proliferative energy of paramœcium. In certain of his series which had been maintained for considerable periods of time on a uniform diet and were consequently relatively inactive, the effect of subjecting

[15] *Journ. Exper. Zoöl.* 1904, i, 423.

them for a period of twenty-five to thirty minutes to the influence of such salts as potassium phosphate and potassium chloride, and once more returning them to the original medium was to increase greatly their rate of subdivision for a considerable period exceeding in time one or even two generations of our mouse tumors.

A careful consideration of the theory elaborated above may be made to throw much needed light on the perplexing problems which daily present themselves in studying cancer, more especially in connection with the transplantation of mouse tumors. Enzymes and toxins, like catalytic chemical agents, exhibit, as stated above, marked variations in activity with varying temperatures. Toxins and poisonous chemical bodies are capable at extreme dilution and within very narrow limits of concentration of exerting a marked stimulation upon the cell. We have ample evidence of the existence of a stimulating agent in the cancer cell. If this body is possessed of a chemical or enzymotic nature, it is only to be expected that incubation of the cells containing it, in dilute saline solutions, would lead to its liberation and transformation into a temporarily more active state. Such a stimulus, even applied for an extremely short time, is capable, as shown above, of exerting a prolonged effect on certain organisms. In our experiments the stimulating agent may be said to be present at optimum concentration for producing proliferation in those tumors which on direct inoculation produce yields of 80 per cent. and upwards. Consequently any increase in temperature may be expected to exert destructive action, the stimulating body thereby reaching a degree of activity which would lead rather to the destruction than to the stimulation of the cells. On the other hand, tumors possessed of a less marked proliferative energy, which have grown less rapidly, shown less increase, and yield a smaller percentage on direct inoculation, may be stimulated to produce an optimum effect at a temperature slightly above the normal; whilst those which under normal conditions give extremely small or practically no yield may be stimulated by even higher temperatures, the activity of the relatively small concentrations of the agent in question being so increased

by incubation as to lead to a relatively increased yield after such a treatment.

That chemical stimuli exert an effect only within very narrow limits of concentration and only in the presence of suitable solvents may well account for the extraordinary difficulties sometimes experienced in obtaining successful and uniform transplantation of tumors. It may account for variation in the speed of development of individual tumors in the same series, for spontaneous recoveries, etc.

In conclusion, we may once more draw attention to the fact that those tumors which apparently contain excessive quantities of the body in question, are those which have recently reached maturity, have partially undergone necrosis, and contain relatively large proportions of calcium. The compounds of complex organic bodies with calcium are, for the most part, relatively insoluble as compared with those of potassium and sodium. It is conceivable that this extraordinarily rapid necrosis, without intervention of bacteria, of those tumors derived from the most virulent series associated with a preponderance of calcium over potassium,[16] is a reaction on the part of the organism whereby the active stimulating agent is at least for the time being removed from the scene of action by precipitation.

In such a case it is conceivable that the influence of incubation in a dilute saline solution is to convert the stimulating agent into an active, soluble form capable of exerting an effect upon any susceptible cells subjected to its action.

Such a stimulating agent as that referred to above is obviously not generated spontaneously, and it would be interesting to trace its possible source by observing to what extent the above results can be made to harmonize with the theories generally advanced regarding the origin of cancer. Owing however to the paucity of our present material it would be preferable to postpone the consideration of this question until we have more experimental data at our disposal.

[16] Clowes and Frisbie, *loc. cit.*

STUDIES UPON CALCAREOUS DEGENERATION.*

V.—THE RELATION OF EXPERIMENTAL ARTERIAL DISEASE IN ANIMALS TO ARTERIOSCLEROSIS IN MAN.

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PLATE XXIV.

Since Josué first produced arterial changes in rabbits by means of adrenalin there has been a whole host of experimenters who have repeated and confirmed his work. Although Josué was the first to produce arterial lesions by means of adrenalin, he was preceded by Gilbert and Lion who were successful in bringing about arterial changes, by means of bacteria and their toxins; these lesions they believed simulated arteriosclerosis in man. Sumikawa, too, caused inflammatory changes near the arteries in rabbits, and believed that the vascular changes were identical with those found in arteriosclerosis. However, there is no doubt that arterial changes have been produced with most success by the inoculation of adrenalin. Erb, Baylad and Albarède, Külbs, Fischer, Scheidemandel, and Lissauer have experimented with adrenalin, and have noted the vascular changes. In the main their observations agree, but details of minor importance have been emphasized by some of them. B. Fischer has further found that digitalin when inoculated intravenously produces lesions similar to those caused by adrenalin.

The French workers, Josué and Baylad and Albarède, are most emphatic in maintaining that the intimal changes found in the vessels of experimental animals are of prime importance. The others, with whom I agree, hold that the medial lesions are the principal changes caused by adrenalin. The intimal changes,

* This study was carried out at McGill University, Montreal, under a grant from the Rockefeller Institute for Medical Research.

which are rarely found, are of a secondary nature, or else are brought about by a process quite distinct from that producing the medial changes.

On account of the importance of a proper interpretation of these experimental lesions, and of the relation which they bear to the diseases of the arteries in man, I feel justified in making a histological comparison of the experimental and human lesions. It is impossible to call the macroscopical changes in the rabbit's aorta atheroma or arteriosclerosis unless the histological examination supports this view. I do not doubt that some have been misled in the use of these terms by the naked-eye appearance of the intima, while others have used the term arteriosclerosis in its broadest sense. Should the latter course be adopted, it should be recognized that experimental arteriosclerosis in rabbits is entirely distinct from sclerosis of the aorta in man. Moreover, as I shall point out later, the term atheroma should not be used in connection with the adrenalin lesions.

It is unnecessary for me to give in detail the methods of experimenting on animals, further than to state that my methods resemble those of previous investigators and more particularly those described by B. Fischer. The pure solution of adrenalin chloride, one in a thousand, was injected into the ear veins of rabbits.

Microscopical lesions, such as dilatation of the blood-vessels, hæmorrhages in the brain, and aneurismal distentions of the larger vessels, were noted. The most frequent lesions were situated in the descending arch of the aorta, where white calcified plaques formed the chief naked-eye alterations. The aneurisms were found to be situated in the centres of these white plaques which have been described by some as atheromatous areas. Histological examination shows that these plaques, at an early stage, are situated entirely in the media and that the calcification which occurs here is the secondary result of a degenerative, chiefly fatty, change which takes place in the media. Calcification is never found in the intima, and only rarely are there any intimal changes. The intimal changes which occur are of two kinds: either there is a

local heaping up of endothelial cells at a point where no degenerative changes of any kind have taken place, or there is thickening of the intima underneath the endothelial cells, chiefly due to proliferation of the connective tissue, in which slight fatty changes may occur. Such being the case, the term atheroma must be discarded in describing the experimental arterial lesions in rabbits.

As I have pointed out elsewhere, the primary change in the media, preceding the stage of calcification, is fatty degeneration of the muscle cells, followed by fatty degeneration of the elastic fibres. In the earliest lesions, before any macroscopic change can be noted in the vessels, fine granules of fat are aggregated about the nuclei of the muscle fibres. These granules of fat have an arrangement not unlike that seen in fatty degeneration of the heart muscle. From this stage of degeneration the process advances rapidly so that in a few days the median zone of the middle tunic of the vessels is in an advanced stage of degeneration. Fischer has already noted the rapidity with which the muscle fibres change under the influence of adrenalin inoculation, and hence has applied the name arterionecrosis. Since the degenerative changes are not so rapid as to prevent the appearance of fatty degeneration, and since there proceeds with the apparent necrosis a process of calcification, the name proposed by Fischer is a little misleading. I must grant that sections stained with hæmatoxylin and eosin give a picture of an active necrosis, but with Weigert's stain alone, or combined with Sudan III, the necrotic appearance disappears.

My studies lead me to accept the histological descriptions given by Fischer and others, except, as I have stated, that I find fatty changes in the muscle and elastic fibres preceding the deposit of the calcium salts. The mode in which these calcium salts are laid down has also been discussed in full in another paper, but as regards the sites of the calcium deposit I wish to speak more fully.

In sections stained with hæmatoxylin and eosin, it is seen that the lesion lies in the middle zone of the media and that in the early stages the muscle fibres become granular and lose their nuclei. With this degeneration of the muscle fibres, which must

necessarily be accompanied by softening of the tissue, the elastic fibres are packed more closely together, leaving less room between them for the muscle cells. At these points, where there is a loss of the muscle elements, the vessel wall is distinctly weakened, and partially gives way, leading to small aneurismal sacculations. In advanced lesions these aneurismal dilatations are the most marked macroscopic changes that are found.

When the muscle cells in the media have undergone fatty degeneration, the condition advances, until the cells become fairly loaded with the fat granules. Nuclear degenerations then become apparent and soon the cells die, leaving only their outline marked by the deposit of the fat granules which were in them. These fat granules do not coalesce but remain as isolated particles. Passing through the stage of fatty acids and soaps[1] they are converted into calcium compounds of the fatty acids, and hence the calcium deposit occurs as a fine sandlike material distributed in the sites of the former fatty granules. Following closely upon this change, the elastic fibres also become fatty, as Jores has observed, and go through the same process of calcification as the muscle cells. There is now a fine granular deposit of calcium at the site of the muscle fibres and rigid zig-zag calcified elastic fibres lying between them. This picture is not seen in the advanced stages of the calcification of aorta in rabbits, for the calcium salts have coalesced more or less to form larger masses, so that the former histological structure of the media is entirely lost. In no instance of experimentally calcified vessels in animals has primary calcification of the intima been noted. Occasionally I have seen very advanced experimental arterial disease in which the calcification of the media extended immediately below the intima.

If we are to accept Jores's definition of arteriosclerosis, namely, that the disease consists of a hyperplasia with degeneration of the musculo-elastic layer of the intima as seen in the human aorta, these experimental lesions would not fall in this category. I am not, however, inclined to accept this restricted

[1] See the studies upon calcareous degeneration by the writer. *Jour. of Exper. Med.*, 1905, vii, 633.

definition of arteriosclerosis, particularly as regards the human disease.

Lissauer compares the experimental lesions in arteries of rabbits to syphilitic arterial disease in man, pointing out that syphilitic arteritis attacks particularly the media. He falls short of a proper comparison. The main factor in the experimental lesions is the calcification of the muscle and elastic fibres in the media, and this never takes place in syphilitic disease of the vessels. In syphilitic arteritis the main reaction, consisting of an inflammatory change with new formed blood-vessels, an infiltration of small cells, and later the production of connective tissue, takes place in the outer third of the media. The blood vessels affected by adrenalin, on the contrary, show no reparative reaction in the media, the process being entirely a degenerative one. If a reaction does occur, it is secondary to a medial degeneration, and this reaction consists of a heaping up of the endothelial cells in the intima.

I would point out that although the lesion does lie in the media—and especially in the middle zone of the media—there are no changes in the vasa vasorum. All the hypotheses, maintaining that the action of adrenalin on the vasa in the vessel wall is the prime factor in the production of the necrosis, are based upon general inferences regarding the action of this drug in other parts; the histological appearances give no support to this theory.

A second arterial disease which Lissauer wishes to compare with the vascular changes caused by adrenalin is the so-called neurotic angiosclerosis. He points out that Lewaschew and also Fraenkel found that by injuring or cutting the nerve supply of vessels, they became infiltrated with connective tissue, and that the change lay only in the media. Their results are quite contrary to those of Jores, who found that neither injury to a nerve nor the complete severance of it had any effect on the histological structure of the vessel it supplied. In syphilitic arterial disease, as I have mentioned, and in the neurotic lesion, calcification of the media is never seen.

There is, however, an arterial disease in man which simulates the

experimental arterial degenerations in every respect. This disease I have spoken of elsewhere as Moenkeberg's type of arteriosclerosis. This disease, as Moenkeberg and others have pointed out, affects the vessels of the extremities—the vessels of the muscular type—that is, arteries in which the muscle tissue predominates in the media. From this lesion the clinician makes the diagnosis of arteriosclerosis when he finds the radials hardened like "pipe stems" or "beaded like a trachea." It is now definitely known that this form of arterial disease is distinguishable from arteriosclerosis as we find it in the human aorta. Moenkeberg's type of arteriosclerosis affects the media alone, causing degenerative changes. The intima is affected neither by degenerative nor by reparative changes, or if such changes are present they are secondary to or parts of another disease and not associated with changes taking place in the media. This disease of the media affects the vessels of the extremities—the femorals, tibials, internal and external iliacs, radials, and brachials being particularly susceptible.

Preceding the deposition of calcium salts in the media, there are degenerative changes which consist chiefly of fatty degeneration, which is visible to the naked eye, and can be distinctly traced in the media when the vessel is cut open longitudinally. This early fatty degeneration in the media differs from that of arteriosclerosis of the aorta in that the fatty plaques do not stand above the surface of the intima, but can be seen through this membrane. One is convinced by the microscopical picture, that in the early stages the muscle cells undergo a fatty degeneration characterized by the deposition of the finest fat globules. The calcium salts, too, are found to be deposited as fine sandlike granules in the degenerated muscle fibres. In the early stages of degeneration, calcium granules are found in double wedge-shaped aggregations lying between the elastic fibres, and this grade of calcification is not to be recognized by the naked eye. However, with the constant accumulations of fresh calcium deposit, the granules lie very close together and then coalesce. At this stage, when the calcium granules are becoming densely aggregated, we find that the vessel wall decreases in thickness; there is a thinning of

the media with a bulging outward of the wall at this point. Distinct multiple aneurismal dilatations are of common occurrence in these vessels, so that the outer contour of the vessel is quite irregular. From the inner surface of the vessel it is seen that the calcified areas form depressions which are lined by smooth and unchanged intima. Microscopically, the elastic fibres are seen to be stretched into straight lamellæ and no longer show the wavy character found in healthy vessels. The elastic fibres, too, are calcified and are lying more closely together, although in advanced cases this is difficult to distinguish, as the calcium deposit in the muscle and elastic fibres have run together into a solid mass. In consequence of the constant tension and stretching of the elastic fibres, they have become calcified in the position that they had in these vessels, and thus we find the straight calcified laminæ. Where calcification of elastic fibres has advanced beyond the general area of calcareous degeneration, and, too, where contractile muscle fibres have persisted between them, these elastic fibres have taken on a zig-zag outline due to fractures.

Microscopically one can demonstrate that the running together of the calcium granules takes place in the middle of the media, and that at either end of such a mass are outrunners of calcified elastic fibres and granular degenerated muscle fibres. The internal elastic lamina is stretched as it passes over the calcified and aneurismal portion of the media.

The intima occasionally shows some reaction over the areas of medial degeneration, the endothelial cells being heaped up. This intimal change is, however, entirely secondary and has nothing to do with the primary cause of the medial change, and further is not a necessary accompaniment of the medial degeneration.

Moenkeberg holds that the adventitial changes are in great part confined to thickening of the intima of the vasa vasorum, but points out that this limitation is not a constant feature in the disease. There are some who claim that the degeneration of the media is due to occlusion of the vasa vasorum causing poor nutrition of the media. However, the changes in the vasa vasorum are too inconstant to allow us to draw any definite con-

clusion, and, moreover, it is very hard to estimate the amount of change in these small arterioles with no definite wall, especially when they are collapsed and empty.

Pure medial calcification is commonly found as an accompaniment of advanced age, occurring most frequently after the age of fifty. But, as Marchand has pointed out, it occurs also in young individuals. The disease affects males more frequently than females, the ratio being about six to one. It is further of interest that medial calcification occurs in the vessels of those extremities which are most active; thus in right-handed individuals it is found more pronounced on the right side, while in those whose duties keep them constantly on their feet the femorals are most affected. Again, the disease is particularly common among the laboring classes.

When the experimental lesion in animals and the medial arteriosclerosis in man are compared, they present great similarity in their anatomical, histological, and, it may be, their etiological characters. In each case the essential lesions are confined to the media, the changes in the intima being of a secondary nature or else the result of a different disease. In both lesions the process in the media rapidly leads to calcification of the muscular and elastic elements, fatty change preceding the deposition of the calcium salts. Aneurismal dilatations at the sites of the calcium deposits in the media often occur with both lesions, and in the dilated part the vessel wall is much thinned as the result of the loss of muscular elements and the packing together of the elastic fibres.

Both diseases are brought about or accompanied by an increased blood pressure; in the one it is artificially produced by the use of adrenalin and involves the general circulation, while in the other it is localized in parts, the extremities, where increased work, straining and constriction of the muscles, lead to heightened pressure. Whether this heightened blood pressure acts directly on the vessel walls to cause the medial degeneration, or whether by the stretching of the arteries the vasa vasorum are in part or wholly pinched off, leading to focal necrosis in the media, is not wholly clear. Certainly from the focal character

of the experimental lesions and from the annular arrangement of the degeneration in the vessels of the extremities in man, I should feel very much inclined to think that the vasa played an important rôle in the production of these changes. However, we must not lose sight of the fact that in the experimental animals the toxic character of the substances inoculated may be an important factor in the production of the lesions, as Fischer has pointed out.

Both of these lesions must be distinguished from the intimal form of arteriosclerosis as found in the aorta, and the presence of one condition does not indicate the presence of the other.

Several clinicians have made the observation that when pure medial arteriosclerosis is present in the vessels of the extremities without any changes in the aorta there is no hypertrophy of the left heart, while, on the contrary, if the aorta is affected with arteriosclerotic changes the heart shows hypertrophy. This fact suggests that while the aorta is healthy and elastic, it relieves, to a great extent, the increased blood pressure which would naturally follow the hardening of the peripheral vessels, while, on the other hand, if the elasticity of the aorta is destroyed, the increased pressure is directly transferred to the heart, leading to hypertrophy of the organ. The experimental hardening of the walls of the aorta apparently confirms this view, since hypertrophy of the heart frequently occurs with it.

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Fig. 1.

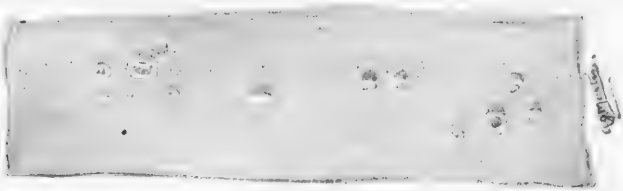

Fig. 2.

Fig 3.

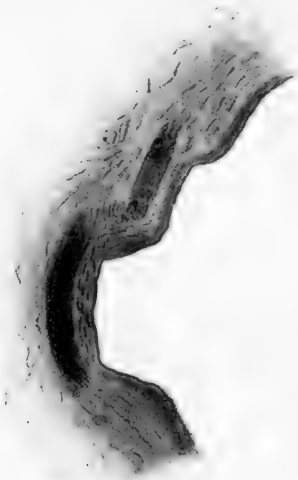

Fig. 4.

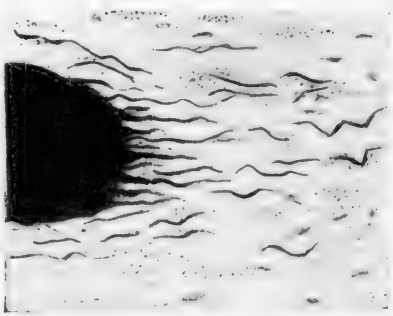

Fig 5.

Pearce and Stanton.—*Trans. Assoc. of Amer. Phys.*, 1905, xx, 513.
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EXPLANATION OF PLATE XXIV.

Fig. 1.—Femoral artery (natural size), showing pittings and small aneurismal dilatations in the areas of calcification of the media.

Fig. 2.—Aorta of rabbit (enlarged x 2), after injections of adrenalin, showing small calcified plaques with depressions and aneurismal bulgings in their centres.

Fig. 3.—Femoral artery (low power, Leitz obj. 3, ocular No. 2), showing calcification of the media with little change in the intima other than splitting of the internal elastic lamina.

Fig. 4.—Aorta of rabbit after injections of adrenalin (low power), showing calcification in the media with no change in the intima. Vessel wall shows aneurismal dilatation.

Fig. 5.—Femoral artery (high power), showing densely calcified media, along with a less intensely calcified area in which the elastic and muscle fibres are in the early stages of calcification.

THE PATHOGENESIS OF EXPERIMENTAL COLITIS, AND THE RELATION OF COLITIS IN ANIMALS AND MAN.

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There is no longer any real doubt that in the group of dysentery bacilli we possess the essential causes of the dysenteric inflammations of the intestine in man. This general statement is justified notwithstanding the facts that in man a class of inflammations of the intestine is caused by pathogenic amœbæ, and an indefinite group of intestinal inflammations, due to no common cause, is occasionally met with. The morbid conditions produced, on the one hand, by pathogenic amœbæ, and, on the other, by irritating foreign bodies, the bacteria of food poisoning and the salts of heavy metals, are, taken together, not very infrequent; but as compared with those caused by the dysentery bacilli they are relatively inconsiderable.

The experimental studies of the past ten or twenty years have shown that a considerable number of diverse poisons produce, in animals, inflammations of the intestine. Some of these poisons are of known definite composition, as the salts of mercury and some other metals; others are of indefinite nature, so far as our present knowledge reaches, as, for example, the peculiar poison contained in putrefying fluids. A third class of poisons of somewhat more definite nature, capable of causing inflammations of the gut, are of vegetable origin: these are the toxic products of bacterial growths, ricin, abrin, etc. While the precise chemical nature of these toxic bodies is still unestablished, the poisons themselves are sufficiently defined to permit of their employment under uniform conditions of experiment. The present paper deals with the effects produced on rabbits by inoculation, chiefly, of a toxic product obtained from the Shiga dysentery

bacillus, and a study of the manner in which the peculiar effects are brought about. In the course of the investigation it was found necessary to resort to the use of some other poisons, *e.g.*, corrosive sublimate and ricin, in order to bring out certain facts which the use of dysentery toxin had failed to establish, or to establish, beyond doubt, certain deductions drawn from the experiments with the dysentery toxin. The results of this experimental study are presented here because of their bearing upon the pathogenesis of colitis in man.

Two main groups of dysentery bacilli are now recognized: the "Shiga" and the "Flexner" groups. The first agree with the bacillus first isolated by Shiga from cases of Japanese dysentery; the second isolated by one of us (Flexner) from cases of Philippine dysentery. The two groups possess in common the capacity to set up dysentery in man. No profound distinction in this power can be drawn between the bacilli. The disease which they cause appears in sporadic, endemic, and epidemic forms. In the case of young children it would appear, from our present knowledge, that the Flexner type of dysentery bacillus causes much more frequently than the Shiga type certain varieties of ileo-colitis. The cultural distinctions between the two groups are considerable: the Shiga type of bacilli has a restricted power to ferment the sugars, while the Flexner type has a rather wide capacity of fermentation. The two groups are also separated by their agglutination reactions: they are less widely separated in respect to their reactions to immune sera. On the other hand, they are sharply separated by their capacity to yield, in fluid cultures, soluble toxins. The Shiga type of bacillus readily produces a soluble toxin, the Flexner variety either not at all or in minimal quantities. Shiga bacilli grown on solid culture media, killed at 60° C. or with chloroform, still yield, when suspended in water or salt solution, a toxin which goes over into the fluid. The Shiga bacilli easily undergo autolysis through which the toxin is liberated. The Flexner bacilli do not yield by autolysis, any more than in fluid culture, a toxic body to a surrounding fluid medium.

As tested on certain small laboratory animals—young guinea-

pigs, for example—the two types of bacilli are about equally toxic. This toxicity does not depend, necessarily, upon multiplication of the bacilli, for the dead micro-organisms are toxic. Hence the Flexner type of bacillus must yield in the animal body an active toxic product. As tested on rabbits, however, the Shiga type of bacillus is more toxic than the Flexner type. The rabbit is, indeed, very sensitive to the action of the Shiga dysentery bacillus or its toxin. The easily demonstrable effects are exerted upon the central nervous system and upon the intestine. Two kinds of intestinal lesions occur either separately or combined. The more common lesions appear in the small intestine: the gut is dilated, the vessels are injected, the Peyer's patches swollen and congested, and a faintly greenish-yellow glutinous or serous fluid fills the lumen. Although occurring less frequently the lesions in the large intestine are much more striking. The part of the large gut affected is, almost exclusively, the dilated cæcum and appendix. Rarely is the colon involved. The lesions vary in intensity: the coats of the gut are greatly thickened by inflammatory œdema, in which case the mucosa is yellowish-white and thrown into deep folds and corrugations, or with the œdema more or less hæmorrhage may be associated. At another time the transverse folds of mucous membrane are affected chiefly; they are swollen, the edges are hæmorrhagic, and pseudo-membrane is scattered over the surface. Or, again, the transverse folds are greatly and the intervening mucosa is less affected, and patches of swollen and hæmorrhagic mucous membrane, covered with a false membrane, appear upon and between the folds. The hæmorrhage may extend into the serous coat. It is impossible, in a brief space, to describe all the variations of appearance of the affected mucous membrane. There is, however, in many cases striking similarity in the lesions, as regards location and general pathological appearances, with the early diphtheritic dysenteric lesions in man. The masses of lymphoid tissue at the ileo-cæcal valve and in the appendix are invariably swollen in the rabbits, showing the large intestinal lesions, and frequently they are beset with small hæmorrhages. The mesenteric lymphatic glands are always swollen, œdematous, and congested.

There are considerable variations of susceptibility to the dysentery toxin among the rabbits. A part only of the rabbits which succumb to the toxin develop the nervous and large intestinal lesions. A given lot of toxin will cause in certain rabbits the nervous and intestinal lesions, and in still other rabbits transitory illness from which they recover, or death without visible lesions. Neither does there appear to be a marked relation between dosage and lesions, or even death. Of a given toxin 0.01 cubic centimeter may be fatal to one series of rabbits while another series may survive 0.1 cubic centimeter of the same poison, the weights of the rabbits having been about equal. Larger doses usually kill, but the period of survival varies considerably. The symptoms which follow the inoculations are diarrhœa, and paralysis and convulsions. The diarrhœa is sometimes bloody. The paralysis begins in the upper extremities and extends, at times, to the lower extremities. It may be incomplete. The animals may survive for days after the extremities are paralyzed. They lie on the side with the head drawn back, and in opisthotonic position. The convulsions are irregularly intermittent, and may be excited by disturbance of the animal. Towards the end of life the paralysis of the extremities becomes more marked. In general appearance these animals resemble rabbits in the paralytic stage of rabies. Although rabbits may survive many days after the onset of the paralytic symptoms, which may themselves be several days in developing, yet in some instances the symptoms of paralysis may appear within twenty-four hours and the animals survive this appearance only a few hours. Speaking generally the larger doses of toxin —0.5 to 1 cubic centimeter—produce marked symptoms and cause death earlier than the smaller doses.

The dysentery toxin which we are considering was first obtained by Conradi, whose results were briefly mentioned by v. Drigalski[1] and later were fully described by himself.[2] The method employed by Conradi consisted of aseptic autolysis of the cultures. Of his preparations 0.1 cubic centimeter was fatal,

[1] V. Drigalski.—*Veröffentlichungen der Medizinalabtheilung des Königlich Preussischen Kriegsministeriums, Berlin, 1902, Abt. 2, 107.*

[2] Conradi.—Ueber lösliche durch aseptische Autolyse erhaltene Giftstoffe von Ruhr- und Typhusbazillen. *Deutsche med. Wochenschr.*, 1903, xxix, 26.

the symptoms preceding death being diarrhœa, collapse, and paralysis. In four rabbits which survived longer than 48 hours Conradi noted the appearance of diphtheritic inflammation of the intestine with which ulceration was associated. Neisser and Shiga[3] confirmed Conradi's findings except that they noticed chiefly the lesions of the small intestine in the poisoned rabbits. The number of authors who have prepared and studied the action of the dysentery toxin is now considerable. Vaillard and Dopter[4] found that when the rabbits succumbed to small doses in from 18 to 24 hours they exhibited the lesions of the small gut, and when larger doses were administered subcutaneously the lesions of the large intestine appeared. Rosenthal[5] and Todd[6] studied toxins obtained by growing the dysentery bacillus in bouillon for several weeks; up to six weeks the filtered cultures gave rise in rabbits to the intestinal and other lesions. Todd does not seem to have met with the inflammations of the large intestine. Kraus and Dörr[7] worked with filtrates of ten-day-old bouillon cultures and with saline extracts of fresh cultures of the dysentery bacilli. Both were toxic. In their opinion no autolysis is required since the living bacilli yield the toxin by extract tion. They do not mention whether the characteristic symptoms and pathological changes occurred in their experiments. Lüdke[8] applied the method of Rowland and Macfadyean, namely grinding the bacilli under liquid air, which operation was followed by extraction with salt solution, to the preparation of a toxin. The product was fatal to rabbits, causing, however, chiefly the lesions of the small gut and only inconsiderable and rare lesions of the large intestine. Lüdke regards the poison as of the nature of endo-toxin, and he failed to obtain a true toxin by growing the bacilli of dysentery in various media. The exudate which is found in guinea-pigs after intraperitoneal injections of dysentery bacilli is, when free of all cells, still toxic. Kikuchi,[9] who described the symptoms produced by this substance, mentions paralysis but not lesions of the intestine.

There is no doubt that all who have studied the dysentery toxin were dealing with the same substance, and the differences in effect met with depend largely upon dosage and the variable re-

[3] Neisser und Shiga.—Ueber freie Receptoren von Typhus- und Dysenteriebazillen und über das Dysenterietoxin. *Deutsche med. Wochenschr.*, 1903, xxix, 61.

[4] Vaillard et Dopter.—La dysenterie épidemique. *Ann. de l'Inst. Pasteur*, 1903, xvii, 486.

[5] Rosenthal.—Das Dysenterietoxin (auf natürlichen Wege gewonnen). *Deutsche med. Wochenschr.*, 1904, xxx, 235.

[6] Todd.—On a Dysentery Toxin and Antitoxin. *Jour. of Hygiene*, 1904, iv, 480.

[7] Kraus und Dörr.—Ueber experimentelle Therapie der Dysenterie. *Wien. klin. Wochenschr.*, 1905, xviii, 1077.

[8] (a) Lüdke.—On the Dysentery Toxin. *Jour. of Path. and Bact.*, 1905, x, 328.

[8] (b) Lüdke.—Ueber die Gewinnung von Dysenterietoxin. *Berl. klin. Wochenschr.*, 1906, xliii, 3 and 54.

[9] Kikuchi.—Untersuchung über die Shiga-Kruseschen Dysenteriebazillus. *Archiv. f. Hyg.*, 1905, lii 378.

action of the rabbit to the injections. These animals succumb chiefly to the nervous poison (neurotoxin) which produces in the brain visible small hæmorrhages and in the spinal cord softening of the gray matter with and without visible hæmorrhages. The upper segment of the cord tends chiefly to be affected. The small intestinal catarrh is almost constantly met with, and the congestion of the blood-vessels beneath the serosa in the large bowel probably indicates that this portion of the intestine is also commonly affected by the slighter lesions. The discrepancies in the literature as regards the nervous and intestinal symptoms and lesions probably depend upon the different manner of their manifestations and the ease with which the former are detected. The absence of intestinal lesions in Kikuchi's experiments, does not depend upon a fundamental difference of action of the exudate and the autolysed poison, for in some of our experiments in which an exudate from the guinea-pig was employed, the intestinal lesions were obtained. Dysentery toxin is a stable substance as it retains its activity in sterile solution under toluol for many weeks, and resists the growth, as we found, of the common moulds. The slighter toxicity of old bouillon cultures would indicate that under certain conditions the toxin tends to depreciate in strength.

There is general agreement among those who have given special attention to the subject that the Flexner type of dysentery bacillus does not yield a soluble toxin. The only dissenting experience is that of Vaillard and Dopter[10] who claim to have obtained identical results with all types of the dysentery bacillus. Since it is not expressly stated that a mannite-fermenting dysentery bacillus was employed it is safe to assume that one of this group was not among the types studied by them.

For preparing the dysentery toxin we used a culture of the Shiga bacillus which had been passed through a series of guinea-pigs to maintain its virulence. The fatal dose for a guinea-pig of 200 to 250 grams was one oese, death taking place in less than twenty-four hours. The cultivations were made upon agar on large flat surfaces, upon which the bacilli grew for twenty-four hours. The growth was washed into sterile water or 0.85 per

[10] *Loc. cit.*, p. 488.

cent. salt solution, the organisms killed by exposure to 60° C. for one hour, and the suspension kept at 37° C. for varying periods. The toxic solution was obtained by filtration through a Berkefeld filter. The filtrate was preserved in the ice-box under toluol. The injections were made intravenously in rabbits.

Although aside from our main theme, which was to ascertain the manner in which the intestinal lesions in rabbits were produced, a series of experiments on the comparative toxicity of living, dead, and autolysed cultures was made. Our results do not uphold Kraus and Dörr who state that an active toxin can be obtained without autolysis, and they indicate, further, that the separated toxin is more effective than the same quantity of poison contained within the living or the dead bacilli.

TABLE I.

Series No.	Weight in grams	Amount injected	Nature of material injected	Results
26	2000	0.1 c.c	living suspension	dead after 10 days
27	1620	"	"	lives
28	1920	"	suspension after 1 hr. at 60° C.	lives
29	1270	"	suspension after 1 hr. at 60° C.	dead after 5 days
30	1150	"	suspension autolysed 3 days	dead after 11 days
31	1150	"	suspension autolysed 3 days	dead after 2 days

TABLE II.

Series No.	Weight in grams	Amount injected	Autolysed — days	Results
23	1610	0.5 c.c.	2	lives
24	1570	"	3	dead after 2 days
25	1740	"	4	lives
32	2470	"	5	dead after 5 days
33	1270	"	6	" " 5 "
34	1300	"	7	" " 2 "
35	1300	"	8	" " 2 "
38	1700	"	9	lives
39	1310	"	10	dead after 3 days
66	1160	1.0 c.c.	15	" " 2 "
75	900	"	27	" " 6 "

Since Conradi believed that the toxin may be weakened by allowing the autolysis to continue longer than forty-eight hours, an experiment was made to determine this point. As Table II shows the toxin is still effective after a period of autolysis extending to twenty-seven days.

The preceding series of experiments serve also to confirm the statement already made, namely, that individual peculiarities play an important part in determining susceptibility to the poison. Of several rabbits the larger may succumb and the smaller escape, or those receiving the smaller doses may die and those a larger dose survive. But where very large doses, from two to five cubic centimeters, are injected, the animals much more rarely survive; and under these circumstances the animal receiving the largest dose usually succumbs the earliest.

The tendency of the diphtheritic lesions of the intestine to confine themselves to the cæcum and colon led early to the notion that retention of the fæces was the chief agent in causing the condition. All the earlier views are summed up in Virchow's paper which appeared in 1863 in which he defined his own belief that fæcal retention is a necessary condition to the production of diphtheritic dysentery. This view now dominates in the pathology of dysentery. It will be instructive to present, in abstract, Virchow's [11] argument in order that a comparison may be made of the conditions present in dysentery in man with those occurring in the experimental disease in the rabbits.

"Dysentery arises from the action of products of decomposition upon the intestinal mucosa already the seat of a superficial catarrhal inflammation. That this is true is shown most clearly by the manner of extension of the dysenteric process. The projecting points—those which come directly and remain longest in relation with the contents of the intestinal mucosa—are first and most severely affected. Rokitansky is mistaken in saying that as a rule the dysenteric process extends from the valve to the rectum, taking on in intensity from above downwards. Generally speaking those portions of the large gut of which the contents remain longest in contact with the mucosa are most severely affected. Those parts are the cæcum, the hepatic, splenic, and sigmoid flexures, and the rectum. Should the intestine present abnormal flexures then the rule holds for them, should the normal flexures be accidentally wanting then this part of the

[11] *Virchow's Archiv*, 1863, v, 348.

gut remains relatively free of disease. In particular it is the projections of the mucous membrane, caused by the anatomical distribution of the muscle tissue, which are affected by preference. Hence the especial susceptibility of the mucosa along the insertion of the three longitudinal muscle bands, and of the transverse folds of the membrane. In that the diphtheritic process establishes itself first on the projecting and later on the intermediate mucosa, there arise those regular geographical figures which indicate the intensity and mode of spread of the lesion. When it is considered that the diphtheritic process does not begin regularly at a definite portion of the mucous membrane, as, for example, the rectum, but that this portion is often least involved while a higher level of intestine is often intensely affected, then the presumption is difficult to set aside that the pathological process is due to some agent which, acting locally, causes the lesions.

"Rokitansky (*Pathological Anatomy*, ii, p. 265) compares with right the anatomical changes in dysentery with those which caustic acids produce; and hence the question arises whether the necrosis is due to a direct chemical change in the tissues caused by an action starting from the surface. Dysentery begins, undoubtedly, as is the case with every superficial inflammation of the mucous membrane, as a simple catarrh, the lesions being met with only after the diphtheritic condition has appeared. Hence it is necessary to ascertain whether the increase from catarrh to diphtheria takes place without the action of local injurious agencies. It may in general be assumed that a simple intestinal catarrh, by reason of the presence of products of decomposition, is converted at those points where decomposition is going on into a diphtheria; and these products are contained chiefly in retained fæcal masses."

In his paper on dysentery published in 1877 Virchow [12] states that diphtheria of the intestine may exist independently of dysentery and instances his findings in some cases of Asiatic cholera of diphtheritis of the intestines. What is of special interest, in this connection, is the statement that the diphtheritic process may involve the ileum, affecting partly the surface of Peyer's patches and partly the villi. "In the latter cases the mucosa was much thickened, firm, and disposed in high transverse folds, making it appear at first as if it were jejunal mucosa.

The increase in our knowledge since these writings has yielded the tangible agents of dysentery and set aside the notion of the action of the products of decomposition, unless, indeed, the toxic constituents of the dysentery bacilli be viewed as such products. Although we have secured this advantage of knowledge, no direct application of it to the pathogenesis of the lesions of dysentery has been attempted. It is clear from the

[12] *Charite Annalen*, 1877, ii, 706.

descriptions of the experimenters with dysentery toxin that the intestinal lesions in rabbits agree very closely with those of human dysentery. The small intestine is found constantly, or nearly so, in a state of catarrhal inflammation, and in a percentage of animals the large gut shows diphtheritis, œdema, and hæmorrhage.

None of the previous experimenters seems to have been impressed with the finer localization of the lesions in the large gut of the rabbit. Aside from the statement that the cæcum is the seat of predilection nothing is recorded. An analysis shows, however, that two main sets of lesions are encountered. In one the parts of the intestine chiefly affected are the transverse folds of the mucous membrane of which the tips or the entire fold may be involved. In some instances the intervening mucosa escapes, in others the pathological process appears there also, often as focalized areas, but sometimes as a more or less diffuse involvement. In the other a diffuse involvement of the cæcum appears to have been present from the start. The former cases present many points of similarity with the appearances described in human dysentery; while for the latter cases it may be said that in the Philippine Islands one of us (Flexner) [13] observed in autopsies upon American soldiers who died of dysentery, diffuse, hæmorrhagic lesions which agree essentially with the diffuse hæmorrhagic condition present in the rabbits.

The importance of this correspondence appears when it is considered that in man the noxious agent reaches the intestine directly through ingested materials, while in rabbits the poison is injected into the circulation and must reach the intestine through its blood supply. The conclusion, in the case of the rabbits, seems sufficiently obvious; the toxic material is secreted into the intestine and produces directly or indirectly pathological effects. But whether in man the noxious material acts directly or through the blood remains as before an open question.

The rabbit is not subject to infection with the dysentery bacillus when cultures are fed the animal; and we have found that

[13] On the Etiology of Tropical Dysentery, *Philadelphia Medical Journal*, 1900, vi, 414.

injection of cultures of the bacilli directly into the duodenum is apparently without effect in this animal. As was to be expected from this result the injection into the duodenum of considerable quantities (up to 10 c.c.) of the autolysed toxin is also without action. The rabbit is not, therefore, subject to poisoning with the dysentery bacillus or its toxic products when they are brought at once into the gastro-intestinal canal. All other forms of inoculation—subcutaneous, intraperitoneal, intravenous—are effective. It may therefore be assumed that direct contact of the bacilli or the toxin with the mucosa of the rabbit's gut cannot lead either to absorption unchanged or act injuriously upon the membrane. Since it was possible that the poison might be altered in the small intestine and reach the cæcum in an inactivated form, an experiment was carried out to ascertain whether direct contact of the toxin with the mucosa of the cæcum would lead to a different result. A rabbit was starved and given magnesium sulphate per os to empty the bowel. The peritoneum was opened and 1.0 cubic centimeter of dysentery toxin diluted with ten cubic centimeters of salt solution was injected directly into the cæcum and allowed to come into contact with the mucous membrane. No immediate effect was noted, and the animal showed, on being killed forty-eight hours later, no lesions of the intestine. The interpretation to be placed upon this experiment is clear, and all the experiments taken together would seem to indicate that the lesions of the gut present in the rabbits given the dysentery bacillus or its toxin, in such way as to bring the poisons into the blood, are caused not by direct effect upon the surface of the mucous membrane but indirectly through the act of elimination of the poison by the intestinal mucosa. In endeavoring to explain the absence of action of the toxin upon the intestine when given by mouth or injected into the lumen of the gut, tests were made on the resistance of the toxin to peptic and tryptic digestion, but no light was obtained. It was found that the toxin is destroyed by peptic digestion (artificial gastric juice) after twenty-four hours' contact, while 0.3 per cent. hydrochloric acid alone is without influence on the toxicity. On the other hand, the toxin

resists tryptic digestion for eighteen hours, but after a longer contact (at the end of three days) it is destroyed. The addition of solutions of erepsin, prepared by Cohnheim's method, to the trypsin does not accelerate the effects.

The toxin is moderately heat stable. Neisser and Shiga found that a temperature of 75° C. reduced greatly its action; Vaillard and Dopter state that the temperature of 70° to 75° C. maintained for one hour is without effect, while at 75° to 80° C. the toxicity is diminished and at 81° C. destroyed. Rosenthal's specimen of toxin was weakened by heating to 70° C. Our results agree with those of Vaillard and Dopter: the toxin studied by us was injured at 80° C. and destroyed at 81° C., those temperatures having been maintained for one hour.

It was noted in some earlier experiments by one of us (Flexner) that when rabbits are injected intravenously with the dysentery bacillus the living bacilli may be found in the bile. Notwithstanding the negative results obtained from direct injection of the bacilli and the toxin into the intestine, it was considered desirable, in view of the possibility of the intestinal lesions being eliminative in nature, to exclude not only the bacilli but also the entrance, possibly, of the toxin into the intestine with the bile. To accomplish this exclusion two methods were employed: the common bile duct was tied; and a biliary fistula was established. After ligating the duct in two places the portion lying between the ligatures was resected. A permanent biliary fistula was made by inserting a special cannula into the apex of the gall bladder and conducting the secretion through rubber tubing to the exterior. The common duct was always tied off. The operations were carefully conducted with all precautions and infection was readily avoided. Table III shows the somewhat surprising results of this series of experiments. While of the ten control animals five developed the characteristic lesions of the cæcum, of the eight animals in which the duct was ligated two only showed similar lesions, and of the ten animals in which a permanent fistula was established only one animal (Number 19) developed lesions of slight degree. As regards rabbit Number 35 it is safe to assume that the small hæmorrhage noted was caused by manipulation of the gut at the operation.

TABLE III.

Series No.	Weight in grams	Amount injected	Death after—days	Result as regards the lesions of the cæcum
		Controls		
81	1555	5mg.powdr.*	2	no lesions
83	1590	"	11	œdema and congestion, no hæmorrhages
5	1300	0.5c.c.toxin	5	marked œdema, no hæmorrhages
6	1630	0.5;0.5;2.0	lives	
8	2100	5.0	2	no lesions
9	2200	5.0	2	numerous hæmorrhages, with necroses
12	2150	2.0	1	no lesions
14	157c	2.0	2	no lesions
16	1540	0.5	4	very extensive hæmorrhage and necrosis
20	2100	5.0	2	scattered hæmorrhages and necrosis
		Tied duct		
82	1510	5mg. powdr.	9	no lesions
84	1605	"	9	œdema and hæmorrhages
1	1240	0.5;1.0;0.5	7	no lesions
2	1500	0.5;1.0	3	no lesions
10	2140	5.0	3	no lesions
11	2140	2.0	1	no lesions
15	1520	2.0	4	œdema, no congestion, no hæmorrhages
17	1740	0.5	4	no lesions
		Permanent biliary fistula		
3	1230	0.5	2	hyperæmia,no focal lesions
4	1530	0.5;1.0	4	no lesions
13	1880	2.0	1	no lesions
18	1860	2.0	3	no lesions
19	2020	0.5	4	slight œdema, no hæmor-
21	2140	5.0	2	no lesions [rhage.
27	2070	10.0	3 hrs.	no lesions
31	2500	5.0	1	no lesions
35	2100	1.0	1	area of hæmorrhage at pt. of cæcum nearest liver
51	2300	5.0	3	no lesions

In discussing these results it is perhaps desirable to point first to their apparent paradoxical nature. While the direct injection of dysentery toxin into the intestine is followed neither by death of the animal nor development of visible intestinal lesions, the exclusion of the toxin from the lumen of the gut by

* Made by drying in thermostat at 37° C. the autolysed culture fluid.

cutting off the entrance of bile tends to prevent the lesions without, at the same time, preventing the fatal action of the poison. The nervous symptoms develop in animals in which the bile duct has been tied or a biliary fistula established. It would be wrong to conclude that the toxin exercised no influence on the body merely because the intestinal lesions were absent, for in a part of the fatal cases the striking lesions of the cæcum do not appear; and the less striking ones of the small bowel might well exist and be followed by recovery. The test of action is, indeed, a lethal result or the development of the nervous symptoms; for although some of the animals injected with small amounts of the toxin recover, yet none of those receiving into the circulation as large doses as may be introduced in the intestine without producing severe symptoms ever survive.

Hence one is brought to the conclusion that the poison which acts injuriously upon the intestine, through elimination, is also excreted by the bile. Indeed, it would agree best with the experimental results to conclude that of all the circulating poison at any one period a fraction only is eliminated by the intestinal wall, while the larger fraction passes out of the circulation through the liver. This larger fraction, entering the intestine with the bile, is then partially or wholly reabsorbed, only to be subjected in turn to the same series of events. According to this view the intestinal lesions are gradually developed by successive acts of excretion. It does not follow that this elimination takes place only in the cæcum, and that the absorption occurs only higher up in the gut. While it seems probable that the chief absorption does occur in the small intestine, the existence of lesions in this part of the intestine may point to it as being also, although in a minor degree, concerned with excretion, while there is nothing to prove that absorption may not also take place in the large intestine. No explanation is at hand for the failure of the toxin to be absorbed when introduced in mass into the small intestine. After this relation of the poisoning to the bile secretion into the intestine became apparent, we attempted, without success, to reproduce some of the symptoms

of the intoxication by mixing the toxin outside the body with rabbit bile and injecting the mixture into the small intestine.

When the bile is drained away a large part of the toxin is lost, but there still remains available, from the multiples of lethal doses injected, sufficient to cause death through the nervous injury. That the chief intestinal lesions should in these experiments be absent is not remarkable. It is surprising, however, to find that the lesions of the large gut occur more infrequently in the animals in which the bile duct is tied off than in animals not operated upon. It is true that the lesions of the small intestine have been present in some of these animals; and from this it has been concluded, first, that some excretion of the toxin occurs in the small intestine, and, second, that the liver holds back, from the blood, the accumulated toxin which it naturally would secrete into the bile. Whether the poison is merely stored in the liver or destroyed there has not been determined.

The experimental results stated, have brought us to the conclusion that dysentery toxin is both excreted by and absorbed into the blood from the intestine. In this respect the toxin is brought into conformity with certain organic (morphia) and inorganic (iron, manganese) substances which are both absorbed and excreted by the gastro-intestinal mucosa. But interesting as this fact taken by itself may be, our special object at present is to show, as far as possible, the extent to which this property of the toxin of being absorbed and excreted by the intestine may account better than the older views, emphasized by Virchow, for the development of the catarrhal and diphtheritic lesions of dysentery in human beings. The bacteriological studies of cases of dysentery in man examined post-mortem have shown the dysentery bacillus to be present throughout the intestinal tract. It is not limited to the large intestine, although it is, possibly, more abundant in the fibrinous exudations. All parts of the mucous membrane of the intestine can be made to yield the bacillus in culture irrespective of whether they are the seat of pathological changes or not. Since the development in any numbers of the dysentery bacillus does not take place

outside the intestinal tract, it must be concluded that the toxin, which causes the lesions and symptoms of the disease, is produced in the gut. It would agree well with the experimental results related to assume that in man the absorption of the dysentery toxin takes place in the small, and possibly also in the large intestine, but that, usually, the chief elimination is by way of the large intestine; and only under unusual conditions, as in the rare cases of diphtheritis of the small intestine, to any great extent by way of the small gut. This general statement of the manner of production of dysenteric lesions, the diphtheritic ones especially, in man does not exclude the possibility that certain secondary causes contribute to determine the extent of the lesions of the large intestine, among which retained fæcal masses might play an important part.

On account of the variable action of the dysentery toxin upon the cæcum of rabbits, the need was felt of studying, by the methods employed in these experiments, more definite chemical substances which provoke eliminative lesions of the intestine. For this purpose corrosive sublimate and ricin were chosen. The lesions of the large intestine which follow the subcutaneous injection of corrosive sublimate in rabbits have been accurately described by Heilborn[14], and those which result from ricin injections into the circulation by Ehrlich[15] and Flexner[16]. Mercury colitis is such a well-known condition that no special description of it is called for, but the fact may be mentioned that the small intestine escapes injury, while the cæcum and the first part of the colon are the seats of hæmorrhage, necrosis, diphtheritis, and ulceration. The most constant and most pronounced lesions are in the spiral folds of the cæcum. The striking lesions in ricin intoxication are general hyperæmia of the the small intestine associated with an excessive secretion of serum and mucous, and swelling of and punctiform hæmorrhages into the Peyer's patches. The lymphatic glands generally become swollen and hyperæmic, and numerous hæmorrhages

[14] *Arc. f. exp. Pathologie u Pharmakologie*, 1878, viii, 361.
[15] *Deutsche med. Wochenschr.*, 1891, 976 and 1218.
[16] *Jour. Exp. Med.*, 1897, ii, 197.

occur in the omentum, mesentery, and serosa of the gut and abdominal wall.

TABLE IV.

Series No.	Weight in grams	Amount of $HgCl_2$	Death after — days	Result as regards the lesions of the cæcum
		Controls		
29	810	0.038	3	lesions of entire cæcum
39	1295	0.06	3	" " "
44	1550	0.03	1	no lesions
36	1030	0.03	1	" "
57	1540	0.015 0.01	4	lesions of entire cæcum
60	1070	0.015 0.01	2	" " "
		Tied duct		
48	1350	0.03	7	lesions confined to ileocæcal region
50	1290	0.03	1	2 small areas of necrosis
56	1310	0.015	1	lesions confined to ileocæcal region
		Permanent biliary fistula		
49	1320	0.03	1	no lesions, except congestion
55	1810	0.015;0.01	3	mod. lesions of entire cæcum, becoming more intense at end of organ toward the appendix
58		0.015; 0.01	2	slight lesions confined to ileocæcal region

In Table IV are given the results of injections of corrosive sublimate into three series of rabbits: (1) into a control series, (2) into a series in which the bile duct was tied and severed, and (3) into a series in which biliary fistula had been produced. Only in the animals with tied bile duct could any lesions be found when death took place within twenty-four hours after the first injection of the mercury. All the animals which lived two days or longer showed lesions, but in the case of the animal with a tied duct which succumbed after seven days, they were slight, while in the two animals with fistulæ, one dying on the second and the other on the third day, the lesions were much less severe than in the corresponding controls. It would therefore appear that mercury, as is the case with dysentery toxin, is reabsorbed after secretion by the bile into the intestine and excreted chiefly by

the cæcum and colon, which portion of the gut shows the severest grade of injury. As in the case of dysentery toxin, the process of elimination is a gradual one.

In order to test this view of the reabsorption of mercury by the bile from the small intestine enterostomy was performed on two rabbits. In the first the small intestine was attached to the abdominal wound at a point about two inches above the ileocæcal valve, after which the intestine was tied off above the valve. After closing the opening in the abdominal wall the small intestine was opened. There was free outward passage of contents. Two doses of sublimate of 0.02 gram each were given subcutaneously on October 17th and 18th. Rabbit dead on October 19th. Fistula opening patent; cæcum showed very few small lesions at the edges of the spiral valves; no lesions of the colon. In making the opening in the intestine of the second rabbit a small cannula was employed. October 16th, operation done; October 17th and 18th each 0.02 gram sublimate injected subcutaneously. Rabbit dead October 19th. The autopsy showed that the opening of the cannula was occluded by a mass of dried intestinal contents; so that no fluid could escape. The cæcum showed hæmorrhages into the spiral valve with superficial necrosis, and hæmorrhagic infiltration of the colon. This second experiment, through the accident of the closure of the cannula, becomes a convincing control for the first one. It may, therefore, be considered as rendered probable that the intestinal lesions in mercury poisoning are caused not necessarily by the mercury present originally in the blood, but by a process of successive elimination and reabsorption of the metal from the intestinal tract in which process the biliary secretion plays a highly important part.

In carrying out the experiments with ricin a sample made by Merck, the toxicity of which was about 0.1 milligram per kilo of rabbit, was employed. The control rabbit, weighing 1420 grams, was given 0.15 milligram of ricin intravenously on January 27th. Death occurred twenty-four hours later. At autopsy the characteristic lesions inclusive of increased fluid in the pericardium and peritoneum, hæmorrhages into the epicardium and omentum,

swelling and hæmorrhage of the superficial and deep lymphatic glands, much swelling of Peyer's patches, increase of mucoid fluid in the small intestine, and enlargement ($\times 2$) of the spleen existed.

In a second rabbit, weighing 1470 grams, a permanent biliary fistula was made on January 26th, and immediately after the operation 0.15 milligram of ricin was injected. Death took place the next day. The autopsy showed slight swelling of and no hæmorrhage in the lymphatic glands, no excess of fluid in the pericardium and peritoneum, no hæmorrhages in the epicardium or peritoneum, slight swelling of Peyer's patches, and contracted (normal) condition of the small intestine. A repetition of this experiment, using twice the dose, was made. The control and operated rabbits were given 0.3 milligram per kilo of body weight. The former succumbed after nineteen, the latter after twenty-two hours. The lesions in the first were very pronounced and of a strikingly hæmorrhagic character. Considerable hæmorrhage had taken place into the mucosa and contents of the cæcum and appendix. In the fistulous rabbit the lesions were more marked than in the first operated animal and much less severe than in the control rabbit. While some of the peripheral lymphatic glands were not perceptibly enlarged, hæmorrhages had taken place into others, and still others were congested. The patches of Peyer were only slightly swollen; but a few small punctiform hæmorrhages occurred in them; the lymphatic tissue of the cæcum was swollen but free of hæmorrhages.

The experiments with ricin leave no doubt that the pathological effect of the poison is greatly modified by permitting the bile to escape from the intestine; and while the fatal outcome is, perhaps, only delayed by the operation, the profound difference in the tissue and vascular reactions indicate that an essential part or effective quantity of the poison leaves the body with the bile. But in addition the experiments prove that resorption of ricin takes place from the intestinal canal, and the lesions met with also point to an excretion by this organ.

The three sets of experiments which have been presented would seem to have important features in common. If it is

permissible to apply the chief facts of them to the interpretation of the production and localization of diphtheritic dysenteric lesions in man, it may be said that these lesions are due to the action of a toxin elaborated by the dysentery bacilli present in the diseased intestine, which toxin is first absorbed, in the main probably from the small intestine, and eliminated chiefly through the large intestine, which suffers injury through the act of excretion. In offering this view of the pathogenesis of dysentery we would add that the injury upon the tissues once inflicted in the manner mentioned, other micro-organisms than the dysentery bacillus doubtless come into action and complicate and increase the pathological effects of the dysentery toxin.

A study of the histology of the lesions of experimental colitis tends to confirm the conclusion that the poison which causes degeneration, necrosis, and inflammation of the cæcum acts not first upon the surface of the mucous membrane, but upon the mucous membrane and the submucosa simultaneously. In the purely œdematous lesions the mucosa may entirely escape noticeable injury, even the surface epithelium being retained in normal appearance. It is, however, usual for the mucous membrane in these cases to show œdema also, especially of the layer next to the muscularis mucosæ. The exudate in these cases is an inflammatory œdema giving rise to fibrin formation in the submucosa; no striking number of polymorphonuclear leucocytes are seen in the tissues although in the veins they may be increased. The blood-vessels are usually congested.

When hæmorrhage is associated with the œdema the mucous membrane is much more altered. The extravasation of blood takes place chiefly in the mucous membrane and usually causes disintegration of the glandular tissue. Associated with the hæmorrhage is, often, necrosis of the cellular elements of the mucous membrane, the necrosis exhibiting itself by hyaline degeneration of the glandular cells, fragmentation, etc. In the cases in which there is much disintegration and necrosis of the mucous membrane, the submucosa shows, besides œdematous swelling, an invasion of polymorphonuclear cells, and thrombosis of lymph-vessels and of the dilated veins. There is no sharp line

between this condition and the one in which the mucous membrane shows coagulative necrosis and thrombi, and the submucosa an abscess-like condition from a great accumulation of leucocytes. Whenever the submucosa is markedly necrotic it is more or less densely infiltrated with leucocytes, which show, moreover, a decided tendency to collect in two layers—one being in relation to the muscularis mucosæ, the other in relation to the circular muscle. Both of these structures may be invaded with leucocytes. On the other hand, the submucosa may show so great an accumulation of leucocytes of the polymorphonuclear variety that the appearance suggests the formation of an abscess and yet the mucous membrane may be intact. The leucocytes show advanced necrotic changes, but the intercellular tissue does not undergo softening. A true abscess is, therefore, not formed.

The tendency of the pathological changes in the mucosa and submucosa to appear with greatest intensity in the spiral folds is clearly seen in the microscopical sections. The lesions diminish in intensity as the level surface of the mucosa is reached. While the entire depth of the mucosa in the full length of the fold may show coagulative necrosis, or hæmorrhage with disintegration or œdema, as the lower margins are approached the lesions extend less in depth and become discontinuous, until at the level of the mucosa microscopical areas, chiefly superficial ones, of necrosis or hæmorrhage appear in the mucosa, and the submucous infiltration is disappearing. On the surface and within the substance of the necrotic mucous membrane a definite fibrinous and leucocytic pseudo-membrane may form. In this and in the adjacent necrotic membrane a massive bacterial development may take place.

While it is not our intention to describe the histological character of mercury colitis it is proper to state that a study of these lesions has shown us that they differ from those caused by dysentery toxin only in intensity. In localization and in character they resemble the severer type of lesions just described.

SUMMARY.

1. The facts described in this paper are intended to bear upon the pathogenesis of colitis in man and animals.

2. The toxin of the Shiga dysentery bacillus is liberated from the bacillus through the process of autolysis.

3. In rabbits the toxin is not absorbed directly in an active form by the gastro-intestinal tract; in man, however, absorption of an active poison does take place from the intestine.

4. The toxin is excreted in rabbits, and probably in man as well by the intestine, chiefly probably by the large intestine, which being injured by the act of elimination, reacts by the development of inflammation, etc.

5. In rabbits the characteristic action of the toxin depends upon the integrity of the biliary secretion into the intestine. When the bile is prevented from entering the intestine, either by ligature and section of the duct or by establishment of a biliary fistula, no lesions whatever of the large intestine appear, or they are inconsiderable in extent.

6. The loss of toxin through a biliary fistula does not prevent in rabbits the lethal effects which are caused, apparently, by a nervous poison. The tying off of the bile duct seems to prevent the passage of the toxin, in amounts sufficient to cause the large intestinal lesions, into the blood. The liver, therefore, in this condition tends to hold back the toxin from the general circulation.

7. The peculiar effects of the toxin on the large intestine in rabbits is not produced at once, but would appear to depend upon successive acts of excretion of the poison by the bowel.

8. The establishment of biliary fistula reduces the intensity of action of corrosive sublimate upon the large intestine in rabbits; and the lesions of ricin poisoning in these animals are also modified by this operation.

9. Dysentery toxin is destroyed by peptic digestion, and also, though probably more slowly, by tryptic digestion. The absence of power of the toxin to cause poisoning in rabbits when it is brought directly into the lumen of the intestine, is not explained by the destructive action of trypsin.

10. The character of the histological changes in the cæcum of rabbits caused by the dysentery toxin points to an action upon the substance and not primarily upon the surface of the intestine.

SOLUTION OF TISSUE WITH ABSCESS.

By EUGENE L. OPIE, M.D.

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In a former publication [1] I have shown that the two types of cells which especially act as phagocytes, namely the polynuclear leucocytes with fine granulation and the large mononuclear phagocyte or macrophage of Metschnikoff, are each characterized by a distinct proteolytic enzyme. That enzyme which is contained in the polynuclear leucocytes and as I have previously shown [2] is manufactured in the bone marrow is capable of causing proteolytic digestion in the presence of a neutral or alkaline reaction and is almost wholly incapable of action when placed in an acid medium. For this enzyme I have suggested the name leuco-protease, since it is characteristic of leucocytes contained in pus and other inflammatory exudates. The large mononuclear phagocytes of inflammatory exudates are especially concerned with the ingestion and destruction of cellular elements; the same cells are found in lymphatic glands adjacent to the site of inflammation and are doubtless identical with those which, for example, are abundant in the spleen lymphatic glands and other situations with typhoid fever. These cells contain an enzyme which is incapable of digesting proteid in an alkaline medium but is active in the presence of a weak acid; I have suggested for this enzyme the name lympho-protease.

The serum of an exudate produced by injecting aleuronat into the pleural cavity of a dog inhibits the proteolytic activity of leuco-protease [3]. The serum of the blood has the same property. Since this enzyme contained in the polynuclear leucocytes is incapable of digesting proteid in the presence of the blood serum its action is limited to these cells;

[1] *Jour. Exper. Med.*, 1906, viii, 410.
[2] *Ibid.*, 1905, vii, 759.
[3] *Ibid.*, 1905, vii, 316.

it can act only upon substances which have been ingested by these phagocytes and thus removed from the influence of the serum. Doubtless the same property of the serum serves to protect the tissues from the proteolytic enzyme contained in the polynuclear leucocytes, for even should it be set free by disintegration of these cells or otherwise its activity would be inhibited. Since lympho-protease does not cause proteolysis in a neutral or alkaline medium, the alkalinity of the blood serum would check its action should it be set free by disintegration of mononuclear phagocytes.

An experiment in which increasing quantities of serum are allowed to act upon the same quantity of enzyme demonstrates very clearly this anti-enzymotic property of the serum. Previous experiments have shown that the cells of an exudate obtained by injecting aleuronat into the pleural cavity of a dog preserve their power to cause proteolysis when dried after treatment with absolute alcohol and ether. Leuco-protease alone is preserved by this method. A weighed quantity of the dry enzyme has been allowed to act during five days at 37° C. upon a measured quantity of diluted blood serum (10 c.c.) heated to 75° C. for one half-hour and thus partially coagulated and denaturalized. The methods employed have been described in previous articles.

Nitrogen in substances uncoagulable by heat has been estimated by the Kjeldahl method and for convenience is represented by cubic centimeters of 1/10 N sulphuric acid.

20 mgr.	powdered	leucocytes	+	coagulated	proteid					24.2 c.c.
20 "	"	"	+	"	"	+ 0.25c.c.	serum			22.15 "
20 "	"	"	+	"	"	+ 0.5 "	"			18.8 "
20 "	"	"	+	"	"	+ 1.0 "	"			10.6 "
20 "	"	"	+	"	"	+ 2.5 "	"			7.55 "

Control, 2.85c.c.

Two and a half cubic centimeters of serum has almost completely inhibited the proteolytic action of twenty milligrams of the powdered cells (by subtracting nitrogen in uncoagulable form contained in the control mixture, digestion is found to be represented by 3.28 c.c.) while the addition of only a quarter of a cubic centimeter materially hinders digestion.

By adding increasing quantities of the enzyme-containing powder to the same quantity of serum it has been found that a given quantity of serum can inhibit the action of only a limited quantity of enzyme.

10	mgr.	powdered	leucocytes	+	coagulated	serum	+ 2.5	c.c.	serum	6.15 c.c.
20	"	"	"	+	"	"	+ 2.5	"	"	6.5 "
40	"	"	"	+	"	"	+ 2.5	"	"	10.4 "
80	"	"	"	+	"	"	+ 2.5	"	"	19.2 "
160	"	"	"	+	"	"	+ 2.5	"	"	25.15 "

Nitrogen in uncoagulable form in coagulated serum + 2.5 c.c. serum is represented by 6.0 c.c.; in 160 mgr. powdered leucocytes, by 2.4 c.c.

The enzymotic activity of the powder used in the preceding experiment is shown by the following test:

20 mgr. powdered leucocytes + coagulated serum	18.9 c.c.
Control	4.3 "

Two and a half cubic centimeters of serum have completely inhibited twenty milligrams of the dried cells but have failed to prevent proteolysis when a larger quantity of enzyme is employed.

In a former publication I have shown that exudates removed from the pleural cavity of a dog one or two days after the injection of aleuronat fail to undergo autolysis because the anti-enzyme of the serum inhibits the proteolytic enzymes of the leucocytes. In exudates removed three days after injection of the inflammatory irritant slight autolysis occurs. Variation in the enzymotic activity of the serum of the exudates doubtless explains the inconstant results of Schutz [4] and of Zak,[5] who failed to find in human exudates any relation between the autolysis of an exudate and the abundance of its cellular elements.

Since an abscess is characterized by softening and solution of tissue it has seemed not improbable that the fluid obtained by centrifugalization from purulent exudates would fail to exhibit the anti-enzymotic action which is characteristic of sterile exudates produced in the pleural cavity by aleuronat, and, doubtless, according to the previously mentioned observations of Schutz and of Zak, of certain human exudates. As the result of the experimental pleurisy produced by aleuronat, at least, the chest wall is uninjured and the pleural cavity, save for a few adhesions, may return to a normal condition.

[4] *Cent. für inner. Med.*, 1902, xxiii, 1161.

[5] *Wiener klin. Woch.*, 1905, xviii, 376.

A sterile purulent exudate was obtained by injecting a small quantity (one cubic centimeter) of turpentine into the subcutaneous or intermuscular tissue of the flank of the dog. At the end of four or five days a large cavity distended with fairly thick purulent fluid is formed. Such purulent fluid contains polynuclear leucocytes in immense number together with mononuclear cells, fat globules, and particles of degenerated cells. Agar-agar inoculated with this purulent exudate has in every instance remained sterile. A measured quantity (5 c.c.) of this exudate was diluted with four times its volume of 0.85 per cent. sodium chloride and after addition of toluol subjected for five days to a temperature of 37° C.

5 c.c. pus at 37° C. for 5 days	12.6 c.c.
Control	7.05 "
Digestion	5.55 "

The following experiment demonstrates that the same purulent exudate is capable of digesting coagulated proteid under the conditions just mentioned.

2.5 c.c. pus + 5 c.c. coagulated serum	17.45 c.c.
Control	6.4 "
Digestion	11.05 "

In order to determine if the proteolysis caused by the purulent exudate is due to the absence of anti-enzyme in the serum of the exudate, cells were separated from the serum by centrifugalization. A cloudy fluid containing globules of fat but no leucocytes was obtained. The effect of this serum upon a given quantity of enzyme preserved as a dry powder was compared with the anti-enzymotic action of the blood serum from the same animal. In the following experiments the dry enzyme was prepared from the cells of a purulent exudate obtained after injecting turpentine into the subcutaneous tissue of a dog. Twenty milligrams of the powdered leucocytes were allowed to act upon coagulated serum in the presence of 2.5 c.c. of serum of the pus from which the dried enzyme was obtained or in the presence of serum of the blood of the same animal.

	Serum of pus	Serum of blood
With 2.5 c.c. of serum	14.15 c.c.	5.15 c.c.

The proteolysis produced by the same quantity of enzyme acting upon the same quantity of proteid without addition of serum is represented by 16.5 c.c.

The following experiment, in which the conditions do not differ from those just described save that the preserved enzyme was obtained from an exudate produced by injecting aleuronat into the pleural cavity, further demonstrates the almost total absence of anti-enzymotic action in the serum from the abscess.

	Serum of pus	Serum of blood
With 1.5 c.c. of serum	17.0 c.c.	9.6 c.c.
With 2.5 c.c. of serum	17.6 "	9.4 "

Proteolysis produced by the same quantity of enzyme alone is represented by 17.95 c.c., the control containing nitrogen in uncoagulable form represented by 3.95 c.c.; 2.5 c.c. of serum of pus contains nitrogen in uncoagulable form represented by 2.1 c.c.; 2.5 c.c. of blood serum, by 1.7 c.c.

A similar experiment in which serum from pus obtained five days after the injection of turpentine was used, confirms those just described. A measured quantity of coagulated proteid was subjected to the action of twenty milligrams of powdered leucocytes in the presence of one and of two and a half cubic centimeters of serum of pus or, for the sake of comparison, of blood.

	Serum of pus	Serum of blood
With 1. c.c. of serum	17.6 c.c.	6.7 c.c.
With 2.5 c. c. of serum	18.65 c.c.	7.35 "

Proteolysis produced by the same quantity of enzyme without the addition of serum is represented by 15.65 c.c., the control containing uncoagulable nitrogen represented by 4.3 c.c.; 2.5 c.c. of serum of pus contains nitrogen in uncoagulable form represented by 3.3 c.c., and the same amount of serum of blood by 1.7 c.c.

When allowance is made for the quantity of nitrogen in uncoagulable form in the various mixtures before digestion, it is evident that one cubic centimeter of blood serum completely inhibits the activity of twenty milligrams of the powdered leucocytes, but more than twice as much serum from the purulent exudate has no inhibitory action.

Since the purulent exudates used in the experiments just described have been obtained by injecting turpentine into the

subcutaneous tissue, it is possible that the absence of anti-enzyme in the serum of these exudates may be due to the action of this substance. In the following experiment the ability of 2.5 c.c. of serum to inhibit the digestion of coagulated proteid caused by forty milligrams of powdered leucocytes was tested in the presence and in the absence of turpentine. For the sake of comparison the same amount of enzyme was allowed to act upon coagulated proteid with and without turpentine in the presence of 2.5 c.c. of blood serum heated to 75° C in order to destroy its anti-enzymotic activity:

	Without turpentine	With turpentine
With 2.5 c.c. heated serum	31.2 c.c.	28.05 c.c.
With 2.5 c.c. serum	5.55 "	5.4 "
Control	3.55 "	4.3 "

Leuco-protease is capable of causing proteolysis in the presence of turpentine and the power of the blood serum to inhibit this digestion is unaffected by turpentine.

It is not improbable that an increasing quantity of proteolytic enzyme set free by disintegration of polynuclear leucocytes has so far overcome the anti-enzymotic action of a limited quantity of exuded serum that the entire pus is capable of active autolysis and has the power of digesting foreign proteid. The limited ability of a given quantity of blood serum to inhibit the action of increasing quantities of enzyme, demonstrated by the experiment already described, gives confirmation of this view. A purulent exudate is characterized by its ability to dissolve fibrin and necrotic tissue because its serum, unlike that of the non-purulent exudate, does not check the activity of the proteolytic enzyme furnished in great abundance by the polynuclear leucocytes.

A SIMPLE METHOD FOR THE PLATING OF ANAËROBIC ORGANISMS.

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Assistant in Bacteriology.

PLATE XXV.

(*From the Bacteriological Laboratory of the College of Physicians and Surgeons.*)

It is not without hesitation that the writer ventures to add another to the already long list of procedures for the separation of anaërobic bacteria. He is encouraged, however, to describe the method given below by the hope that it may prove useful to other workers in the same field by its extreme simplicity and ease of application, and by its economy of time, in that it makes possible the use of the usual plating method in anaërobic studies.

The most satisfactory methods employed heretofore for the separation of anaërobes have depended almost without exception upon the use of a hydrogen generator. This, with its accessory solutions for the removal of impurities derived from the zinc (lead acetate and silver nitrate), is a formidable apparatus, difficult to keep in order and complicated in application. It is hardly necessary to emphasize to those who have worked with anaërobic organisms the desirability of eliminating the necessity for the use of hydrogen.

The most efficient of the methods of anaërobic separation without hydrogen is that of Roux, who used ordinary glass tubes pointed at the ends. The inoculated agar is drawn into these, the ends are sealed in a flame, and practically anaërobic conditions secured. When colonies have developed, the glass is cracked; or, even better, as the writer has found, the entire agar cylinder is pushed out into a sterile Petri dish with a glass rod of suitably small caliber. The method of Roux, while simple and efficient, presents a number of practical disadvantages. The mass of agar used is of necessity very small and unless, therefore, the

dilution of organisms has been extreme, the colonies will be very close together. Again, the convexity of the surface of the agar cylinder renders microscopical study of the colonies very unsatisfactory. The last but chief objection to this method is that there is a considerable absorption of oxygen by the agar during the time in which it is cooling sufficiently to permit its inoculation. While this, of course, is insufficient to prevent the growth of anaërobic organisms, it is enough to mar any conclusion that could be drawn as to the behavior of such organisms under absolutely oxygen-free environments.

The method to be described, the writer believes, besides being a facile one for separation, permits the assumption of absolute anaërobiosis. It is due entirely to the suggestion of Prof. Hiss, who proposed to the writer the simplification for plating purposes of the usual pyrogallic acid methods, that a successful method was devised.

The apparatus used consists of two circular glass dishes, fitting one into the other as do the halves of a Petri dish, and similar to these in every respect except that they are higher, and that a slightly greater space is left between their sides when they are placed together. The dishes should be about three-fourths to one inch in height, they need be of no particular diameter although those of about the same size as the usual Petri dishes are most convenient. The sole requirement necessary for successful plating is that the trough left between the two plates when put together shall not be too broad, a quarter of an inch being most favorable.

Into the smaller of these plates the inoculated agar is poured exactly as is done into a Petri dish in the ordinary anaërobic work. Prolonged boiling of the agar before plating is not essential. When the agar film has become sufficiently hard on the bottom of the smaller dish, the entire apparatus is inverted. The smaller dish is now lifted out of the larger, and placed, still inverted, over a moist surface—a towel or the wet surface of the table—to prevent contamination. Into the bottom of the larger dish, which now stands open, there is placed a quantity (one to two drachms) of dry pyrogallic acid. Into this, over the pyrogallic acid,

the smaller dish, still inverted, is then placed. A strong solution of sodium hydrate is poured into the space left between the sides of the two dishes, in quantity sufficient to fill the

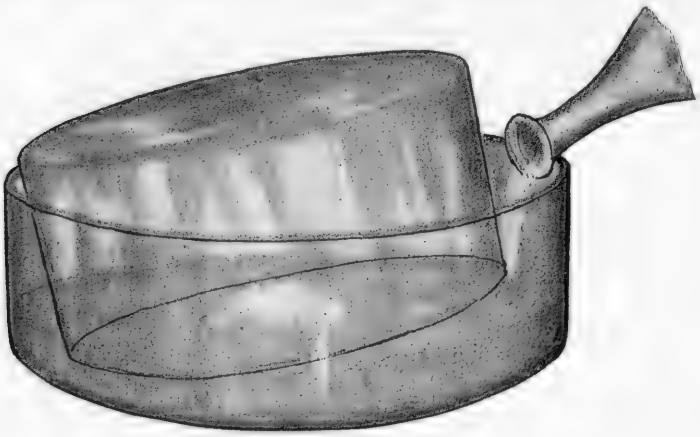


FIG. 1

FIG. 2

receiving one half full. While this is gradually dissolving the pyrogallic acid (and this is the only step which requires speed) alboline, or any other oil, is dropped from a pipette, previously

filled and placed in readiness, into the same space, thus completely sealing the chamber formed by the two dishes.

If these steps have been performed successfully, the pyrogallic solution will at this time appear of a light brown color, and the smaller plate, with its agar film, will float unsteadily above the other. Very rapidly, as the pyrogallic acid absorbs the free oxygen in the chamber, this plate is drawn down close to the other, and the acid assumes a darker hue, which remains without further deepening even after three or four days' incubation.

The accompanying photographs were made of cultures plated in this manner of B. tetani (Plate xxv, Fig. 1) and symptomatic anthrax (Fig. 2). The tetanus culture was separated in this way from a mixed culture containing two other organisms. The colonies thus grown can be studied and fished from in the same way as other colonies grown in Petri dishes.

While the observations the writer has made with this method are limited to the study of three strains of B. tetani and one of the bacillus of symptomatic anthrax, and in both cases to a series not exceeding a dozen platings in each, a few peculiarities of growth have been so constant in their occurrence in all the plates that they are thought worthy of record, especially in the light of the recent work of Smith in America and Tarozzi in Italy, which tends to show that complete anaërobiosis is not the principal factor for the cultivation of the so-called anaërobic organisms, but rather secondary to the presence of the proper nutriment.

In all of the plates observed, the growth has been slow, colonies of the smallest visible size rarely appearing earlier than twenty-four to thirty-six hours in the case of tetanus, or eighteen to twenty-four hours in the case of symptomatic anthrax. These colonies have very rapidly reached their maximum, which in the case of tetanus was rarely larger than about one to two millimeters in size, either in plain agar or in the 1 per cent. glucose agar used. While the initial inhibition in the growth may have been due to oxygen still present in the agar, yet the colonies have remained extremely compact and have even after five or six days' incubation failed to grow more profusely. This appears rather to support the points made by Smith and Tarozzi, since with com-

plete anaërobiosis and ordinary media a less luxurious growth was observed than that obtained by them with a less anaërobic environment but with more attention paid to the constitution of the media.

When fished and examined morphologically, after three or four days' incubation, many of the individuals of these colonies have, by the unevenness of their staining and variations of length, suggested degenerative change. In only two cases in the series observed were spores found in fishing from the colonies, and this in plates where, on account of slowness in the addition of the sealing covering of alboline, the pyrogallic solution was permitted to take on a deep black color (viz., saturation with oxygen). This latter point may in a measure account for the inability of the anaërobic bacteria to form spores under absolutely oxygen-free surroundings. And this again may tend to throw some light on the reason for the absence of the spores in some of the spore-forming anaërobic organisms in the circulation of infected subjects.

In conclusion, the writer takes pleasure in acknowledging his gratitude to Professor Philip Hanson Hiss, without whose suggestion these observations would hardly have been made.

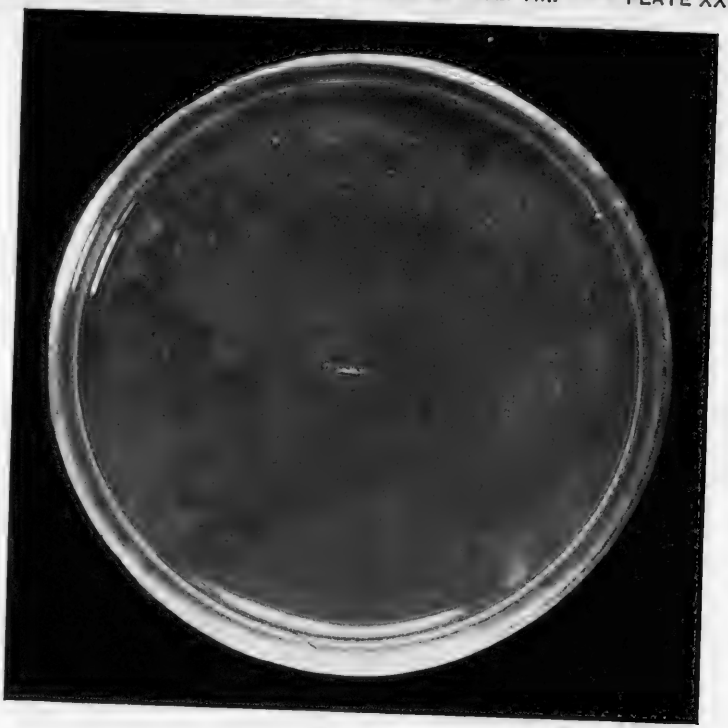


FIG. 1

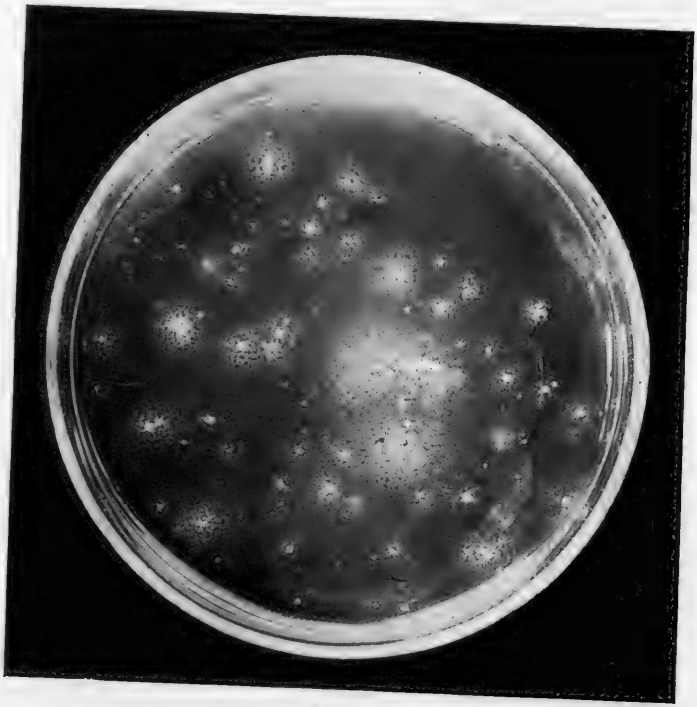


FIG. 2

THE INFLUENCE OF COLLOIDS UPON THE DIFFUSION OF HÆMOLYSINS.

By SIMON FLEXNER, M.D., and HIDEYO NOGUCHI, M.D.

(*From the Rockefeller Institute for Medical Research, New York.*)

Plates XXVI, XXVII, XXVIII, XXIX, and XXX.

The study which forms the subject of this paper was suggested by the experiments made by one of us (Flexner[1]) upon the restraining influence of certain colloids upon the injurious action of bile salts upon the pancreas. The results of the present work not only confirm the view there expressed, namely, that colloidal substances reduce the degree and rapidity of diffusion of certain crystalloids, but they would seem to have even a wider biological bearing. It is for these reasons that they are given, in some detail, in this place. In order that the diffusion of very complex molecules (such as certain hæmolysins, etc.) might be studied it was necessary to devise a method of testing diffusion that would give approximately accurate results without having to resort to chemical analyses which are, indeed, useless with many of these bodies. We found that a suspension of blood corpuscles in certain colloids supplied a useful and accurate means of determining the rate of diffusion of simple and complex molecules which bring about hæmolysis. By reversing the experiment, namely by placing the chemical bodies in the colloids the rate of diffusion into water and saline could also be determined by estimating the quantity of the hæmolytic substance which appeared in these fluids after a given time.

The earlier studies on the influence of colloids upon the rate of diffusion showed, for the most part, that the rate remained unchanged. Graham[2] believed that no inhibition followed from the presence of colloids. He dissolved, among other things, 10 per cent. of sodium chloride in a 2 per cent. agar-agar jelly and overlaid this in a tube with another portion of the jelly

[1] *Jour. Exp. Med.*, 1906, viii, 167.

[2] *Phil. Trans. Roy. Soc.*, 1861, cli, Part I, 183; *Liebig's Annalen*, 1862, cxxi, 1.

minus the salt. At the end of eight days the amount of salt which diffused into the plain agar was found to be the same as that diffused into water. De Vries [3] failed to notice any marked difference in the diffusion of solutions of potassium chromate and copper sulphate into pure water and 4 per cent. silicic acid after a contact of four months. Voigtländer[4] investigated the rate of diffusion of certain simple acids and salts into agar-agar of from 1 to 4 per cent. While the rate of diffusion was at first in favor of the weaker concentration the actual quantities of salts and acids which diffused were about the same in all cases. Reformatsky[5] observed no inhibition of the catalytic reaction of acetate of methyl and hydrochloric acid by a 1.25 per cent. agar suspension; and Levi[6] ascertained that 1.57 per cent. silicic acid, 1 per cent. agar, and 0.6 per cent. gelatin did not alter electric conductivity, lower the freezing point, or inhibit the inversion of cane sugar by hydrochloric acid.

Not all experimenters have found that colloids act in a wholly indifferent manner upon the process of diffusion. Stefan[7] recalculated the values of Graham's experiments and ascertained that the diffusion constant was smaller with agar-agar jelly than with pure water. Chabry[8] found that gelatin and cartilage delayed the action of acids on orcein. While our own experiments were in progress a paper appeared by Kurt Mayer[9] in which the inhibiting action of gelatin, agar, and egg white upon diffusion is described. The bodies employed by him—sodium chloride and potassium chromate—were simple, and his results point to an unmistakable retardation of diffusion by colloids, the retardation being approximately proportional to the concentration of the colloids. With agar the differences are less marked than with gelatin, on account of the wider variations in concentration practicable with the latter substance. Similar results were obtained by Nell[10] who studied the effect of gelatin on the diffusion of a number of chemical bodies. Nell also observed that the electrical conductivity of solutions of copper sulphate are reduced by gelatin when present in amounts of from 1 to 20 per cent.

The number of substances the diffusion of which can be satisfactorily observed by the hæmolytic method is considerable. We studied acids, alkalies, sodium taurocholate, saponin, solanin, cobra venom, and tetanus toxin. In the case of the complex bodies, snake venom and tetanus toxin, the capacity for diffusion of the different constituents, namely, hæmolysins, neurotoxin, tetanospasmin, was also determined.

[3] *Recueil des travaux chimiques de Pays-Bas*, 1884, iii, 375.
[4] *Zeitschr. f. physik. Chemie*, 1889, iii, 316.
[5] *Zeitschr. f. physik. Chemie*, 1891, vii, 34.
[6] *Gazetta chimica italiana*, 1900, xxx, Parte II, 64.
[7] *Wiener Sitzungsberichte*, 1879, lxxix, II Abt., 161 and 215.
[8] *Journal de Physique*, 1888, vii, 115.
[9] *Beiträge z. chem. Physiologie u. Pathologie*, 1905, vii, 393.
[10] *Annalen d. Physik*, 1905, IV Folge, xviii, 323.

The particular points which were noted were: the influence of concentration of colloids upon the rate of diffusion; the time relations of the rate of diffusion; and the comparative degree of diffusibility of different substances.

The diffusible bodies were made to diffuse (1) from saline solution into colloids, (2) from colloids into colloids, and (3) from colloids into saline solution.

METHODS.

In preparing the various solutions and blood mixtures a fair degree of asepsis was observed. Where the experiments extended over any length of time greater precautions were taken. The blood was obtained aseptically and the colloids were sterile. The blood mixtures with agar and gelatin were well preserved at the end of a week or even longer. The experiments were made at room temperature which was about 20° C. The agar suspensions were made with agar-agar which had been thoroughly washed in running water. The agar was dissolved in distilled water, clarified, filtered, and 0.9 per cent. sodium chloride added. The gelatin was dissolved in distilled water, neutralized with NaOH, clarified, filtered, and salt in 0.9 per cent. added.

The highest concentrations of the colloids having been made, the weaker strengths were obtained by the addition of 0.9 per cent. salt solution. Acids, alkalies, and salts were made into normal solutions. These solutions were further diluted according to the hæmolytic strength of the different chemical bodies. In making the mixtures of chemical body and colloid a concentrated solution of the first was added to the second so as to avoid undue dilution of the colloid.

The colloids were pipetted into tubes of 8 mm. to 15mm. diameter, care being taken to prevent flowing over the wall of the tube above the layer. The quantities employed were, usually, 2 c.c. each of colloid and fluid. The colloid column measured from 32 to 33 mm. in tubes of 8 mm. diameter.

Dog and rabbit blood in 3 to 5 per cent. suspensions in 0.9 per cent. salt solution were used. The blood and colloid were mixed at low temperatures after which they were congealed. Ice was used to congeal the gelatin blood mixtures.

The readings were made with a scale one degree of which was 0.7 mm. All the figures were subsequently reduced to millimeters.

In determining the quantity of hæmolytic or toxic body which diffused from the colloid into the supernatant saline the latter fluid was removed at intervals and the hæmolytic or toxic strength determined. These values could be expressed in hæmolytic and in toxic units.

DIFFUSION OF HÆMOLYSINS FROM FLUID INTO COLLOID MEDIA.

There is no difficulty in observing the extent to which the hæmolytic body has penetrated into the blood-colloid suspension since

the originally opaque mixture becomes perfectly transparent as the result of the laking of the corpuscles. The line of penetration is usually sharp and straight, but in some cases it is irregular.

Series I.—Hydrochloric, nitric, oxalic acids, sodium hydroxide, and sodium carbonate in $\frac{1}{10}$ N. solutions were employed. The figures for hydrochloric and nitric acids are almost identical, although the degree of diffusion of hydrochloric is very slightly higher than of nitric acid. The values in millimeters for hydrochloric acid at the different periods (h. equalling hours) are as follows:

	9 h.	18 h.	42 h.	60 h.	84 h.
25% gelatin	4.5	7.7	13.0	16.5	18.2
10% "	5.4	9.1	16.2	20.	22.4

The values for oxalic acid are lower:

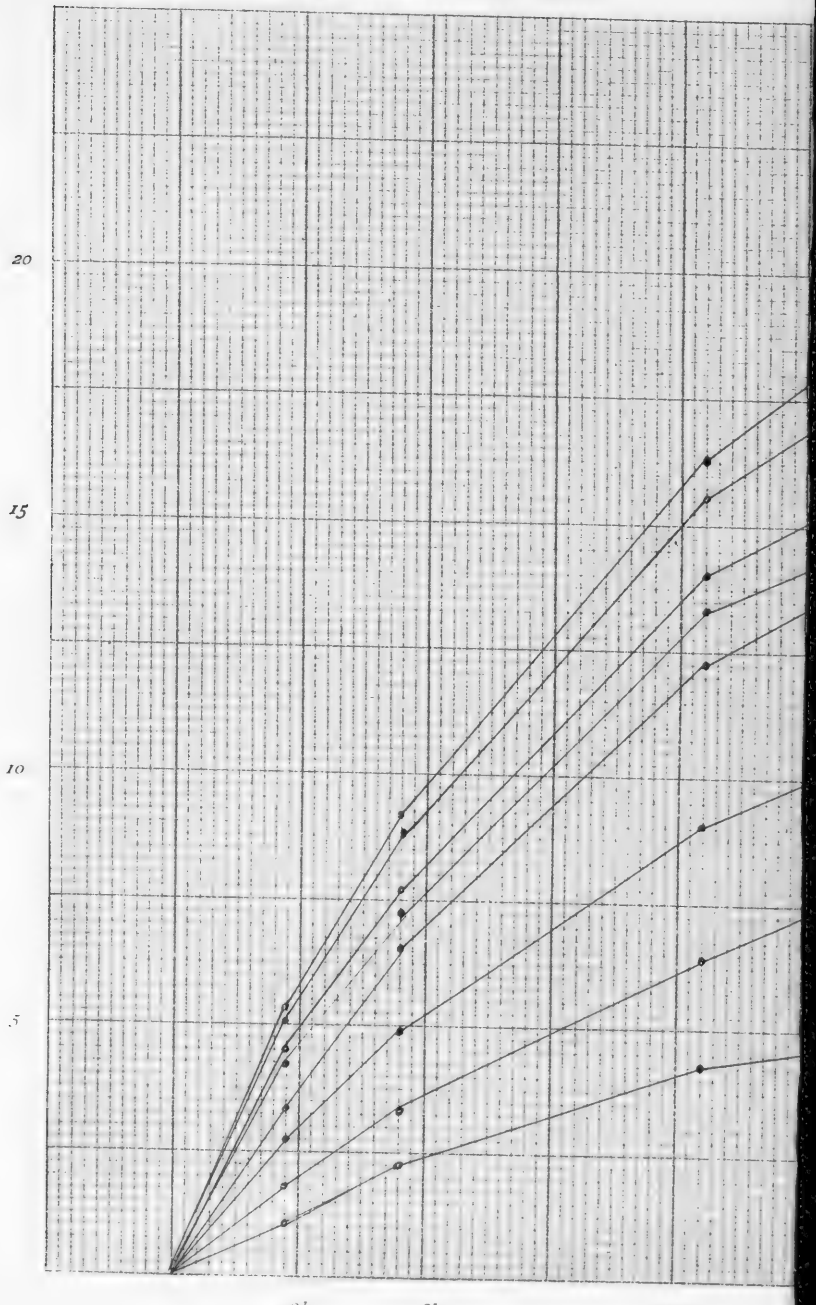
	9 h.	18 h.	42 h.	60 h.	84 h.
25 % gelatin	2.75	4.9	9.1	11.2	12.6
10 % "	3.4	6.3	12.3	14.0	16.8

And the values for sodium carbonate are still lower:

	9 h.	18 h.	42 h.	60 h.	84 h.
25 % gelatin	1.25	2.24	4.2	4.9	5.6
10 % "	1.75	3.3	6.5	8.4	9.8

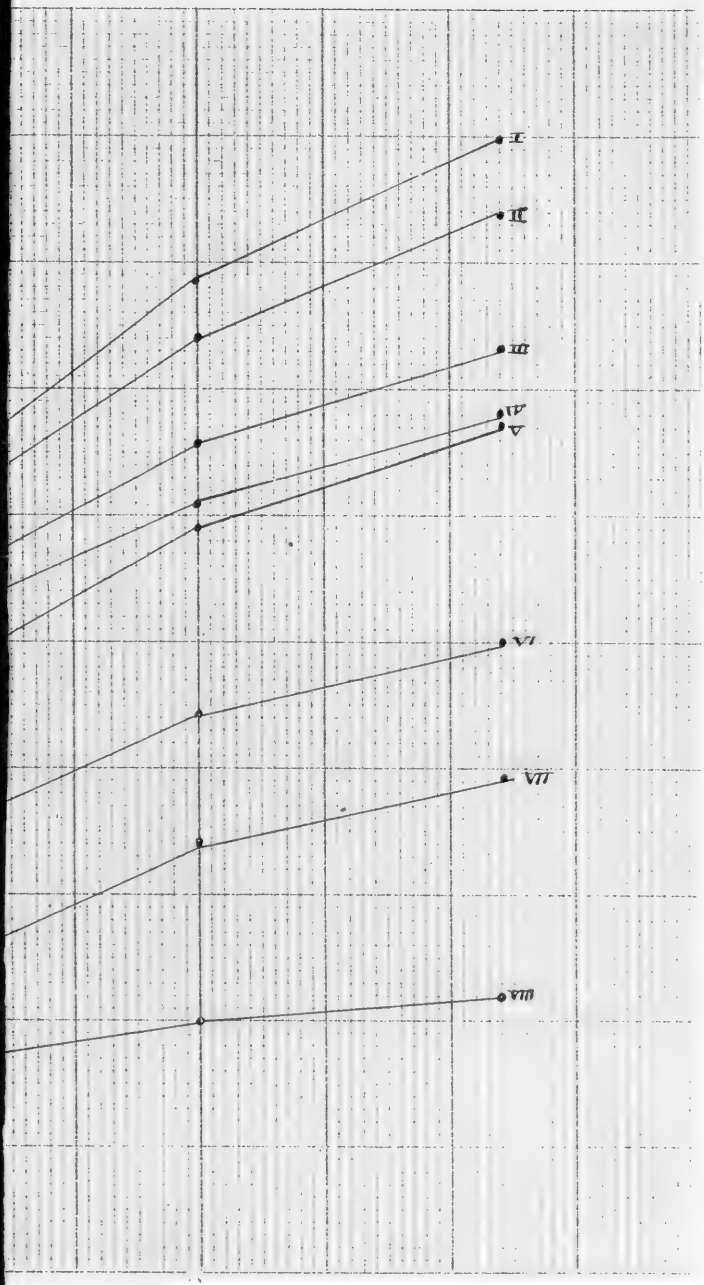
The comparative rate of diffusion into gelatin of these simple bodies can be readily expressed graphically in the form of a curve (Plate XXVI). The influence of concentration of colloid on the rate and degree of diffusion is clearly seen in the curve. Lines I and II indicate the diffusion of hydrochloric acid into 10 per cent. and 25 per cent. gelatin respectively. Lines III and IV indicate nitric acid, V and VI oxalic acid, and VII and VIII sodium carbonate. The black dots show the reading periods and the marginal figures stand for millimeters.

On comparing the diffusion values for 10 and 25 per cent. gelatin the relation can be expressed by the following formula: *The amount of diffusion is approximately inversely proportional to the square root of the concentration of the gelatin.* The deviations from this formula can possibly be accounted for by considerations of experimental error, impurity in the gelatin, and the resistance introduced by the erythrocytes. The velocity of diffusion decreases steadily with increase in time.



1/10 N. HCl { I From saline into 10% gelatin.
II From saline into 25% gelatin.

1/10 N. HNO_3 { III From saline into 10% gelatin.
IV From saline into 25% gelatin.



1/10 N. $C_2H_2O_4$ { V From saline into 10% gelatin. / VI From saline into 25% gelatin.

1/10 N. Na_2CO_3 { VII From saline into 10% gelatin. / VIII From saline into 25% gelatin.

The concentration limits for agar are smaller than for gelatin and consequently the differences in degree and velocity of diffusion are also smaller with that colloid. The strengths employed in the next experiments were 0.5, 1, and 2 per cent. Two examples only, namely of nitric acid and sodium hydroxide, will be given.

HNO_3 $\frac{1}{10}$ N.

	15 min.	30 min.	1 h.	2 h.	3 h.	4 h.
2% agar	5.6	9.5	13.5	18.5	21.3	24.5
1% "	5.8	9.75	13.5	18.75	21.5	24.6
0.5% "	5.95	9.8	13.8	19.0	21.8	24.8

NaOH $\frac{1}{10}$ N.

	15 min.	30 min.	1 h.	2 h.	3 h.	4 h.
2% agar	5.6	9.5	13.5	18.5	21.3	24.5
1% "	5.6	9.5	13.5	18.5	21.5	24.5
0.5% "	5.95	9.6	13.75	18.75	21.5	28.

Series II.—In the next experiments sodium taurocholate, saponin, and solanin were employed. The hæmolytic values were first determined in the ordinary manner. They can be expressed for rabbit blood in the following figures: 0.0001 (saponin), 0.0007 (solanin), 0.007 (sodium taurocholate). The value of hydrochloric acid, under the same conditions was 0.02.

Sodium Taurocholate $\frac{1}{10}$ N. Rabbit Blood 5%

	3 h.	6 h.	12 h.	24 h.	42 h.	60 h.	84 h.	
25% gelatin	1.0	2.0	3.15	4.3	5.6	7.0		1st determination.
		1.2	2.2	3.9	5.6	7.0	8.4	2d "
10% "	1.4	2.6	4.3	6.6	7.8	9.0		1st "
		2.0	3.5	5.6	7.7	9.3	11.2	2d "

Saponin $\frac{1}{100}$ N. Rabbit Blood 5%

	3 h.	6 h.	24 h.	48 h.	
25% gelatin	1.0	2.0	4.2	6.3	1st determination.
		2.4	5.0	7.0	2d "
10% "	1.4	2.9	6.6	9.15	1st "
		2.85	6.0	8.4	2d "

Solanin $\frac{1}{100}$ N. Dog Blood 3%.

	6 h.	24 h.	48 h.
25% gelatin	3.6	7.5	10.5
10% "	4.6	9.0	13.5

The relation which exists in the case of gelatin, between the degree of diffusion and the concentration of the colloid is, as this

experiment shows, the same for certain chemicals of high and of low molecular weight. When agar-agar is substituted for gelatin the differences are less marked, although a difference can still be made out. Taking saponin as an example the following figures are obtained:

Saponin $\frac{1}{100}$ N.

	3 h.	6 h.	24 h.	48 h.	
1% agar	2.45	5.0	9.8	14.0	1st determination.
0.5% "	3.85	8.4	17.5	24.5	" "
1% "	1.7	3.5	8.9	10.5	2d "
0.5% "	1.8	3.8	9.3	11.2	" "

Series III.—The next experiments were made with the complex lysins of cobra venom and tetanus toxin. Two specimens of cobra venom were available: one a fresh solution of 0.4 per cent., one cubic centimeter of which contained for dog blood 2000 minimal hæmolytic doses; and a second solution of the same strength which had been on ice seven months and was ten times weaker. The samples of tetanolysin contained 33 minimal hæmolytic doses per cubic centimeter.

The experiments with dog blood and 0.1 per cent. cobra venom were not wholly satisfactory, since the line of demarkation was not always sharp. The influence of concentration of colloid is, however, shown even in this case.

	3 h.	9 h.	24 h.
25% gelatin	0.84	2.1	6.0
10% "	2.1	5.6	31.5

The next experiment was made with cobra venom, lecithin, and rabbit corpuscles, in which case the diffusion is regular.

Cobra Venom 0.2% 1 c.c., Lecithin $\frac{1}{100}$ N. 1 c.c.

	12 h.	24 h.	42 h.	60 h.	84 h.
25% gelatin	1.7	2.8	4.20	4.9	5.6
10% "	1.8	3.15	4.55	5.55	6.3

The employment of the old cobra venom gave, instead of a curve, an almost straight line.

Passing now to the results with cobra venom 0.1 per cent. and dog blood in agar, definite inhibition in the higher concentration is found to occur.

	6 h.	24 h.	48 h.
2% agar	1.4	5.25	9.1
0.5 "	2.1	7.0	12.6

Cobra venom diffuses into agar more readily than into gelatin. The rate of diffusion into agar is proportional not to the square root of the time but almost directly to the time.

Tetanolysin penetrates gelatin (strength below 10 per cent.) more quickly than it does agar of 0.5 per cent., which makes an exception to the rule.*

DIFFUSION OF HÆMOLYSINS FROM ONE COLLOID INTO ANOTHER.

The next experiments were designed to show whether any further inhibiting effect would be developed if the hæmolysin were enclosed in the colloid. Since the velocity of diffusion is smaller the higher the concentration of the colloid, it was presumable that the introduction of a second colloid medium would further reduce the velocity.

Sodium taurocholate, saponin, solanin, and pyrogallic acid were studied. In the first experiment to be given sodium taurocholate in $\frac{1}{10}$ N. solution was enclosed in gelatin of 10 and 25 per cent., and dog blood of 3 per cent. was suspended in agar of 0.5 and 2.0 per cent.

2% Agar and Dog Blood.

Sod. taurocholate in	6 h.	12 h.	24 h.	48 h.	66 h.
25% gelatin	1.55	2.4	3.15	4.0	5.6
10% "	1.7	2.6	3.6	4.9	7.0
Saline (control)	2.55	3.55	5.15	7.3	9.8

*Arrhenius and Madsen (*Contributions from the University Laboratory for Medical Bacteriology*, Copenhagen, 1902) have applied the method of diffusion into gelatin to a determination of the molecular weight of diphtheria toxin. They ascertained that diphtheria and tetanus toxin diffused into 10 per cent. gelatin; that the former underwent no change in the process and the latter diffused more rapidly than the former. The corresponding antitoxins passed into the gelatin more slowly than the toxins.

Craw (*Proc. of the Royal Soc.*, Series B., 1906, lxxvii, 311) determined that bacillus megatherium lysin passes more slowly through a porous filter impregnated with 15 per cent. than with 7.5 per cent. gelatin.

0.5% Agar and Dog Blood.

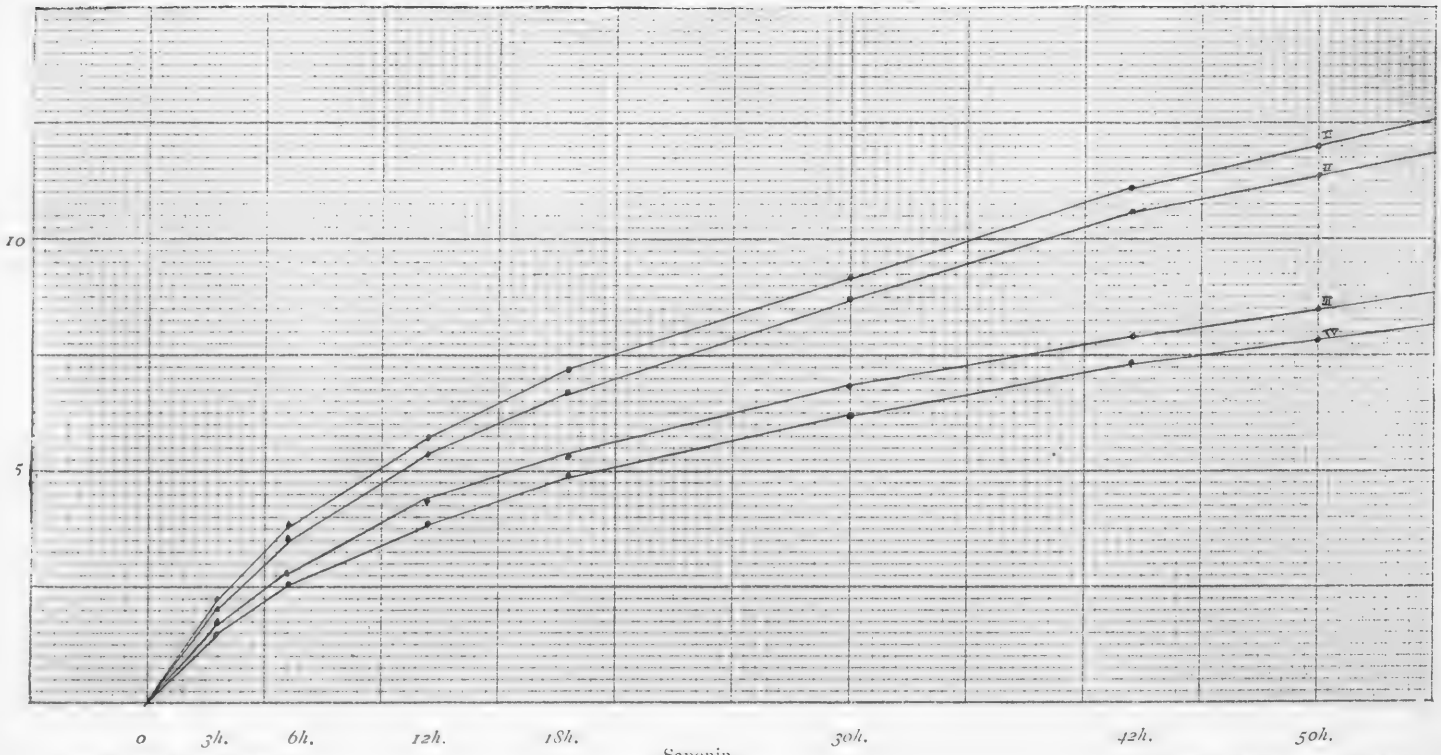
Sod. taurocholate in	6 h.	12 h.	24 h.	48 h.	66 h.
25% gelatin	1.5	2.45	3.15	4.55	5.6
10% "	1.8	2.8	3.8	5.0	7.0
Saline (control)	2.6	3.85	5.2	7.3	9.8

Hence taurocholate of sodium diffuses from 25 per cent. gelatin into agar more slowly than it does from 10 per cent. gelatin. The difference in concentration of the agar has only a slight influence on the rate of diffusion. In a second experiment the salt was dissolved in agar and the blood suspended in gelatin. The influence of concentration (0.5 and 2.0 per cent.) of the agar was very small, and the diffusion from agar was slightly greater than from saline solution (control).

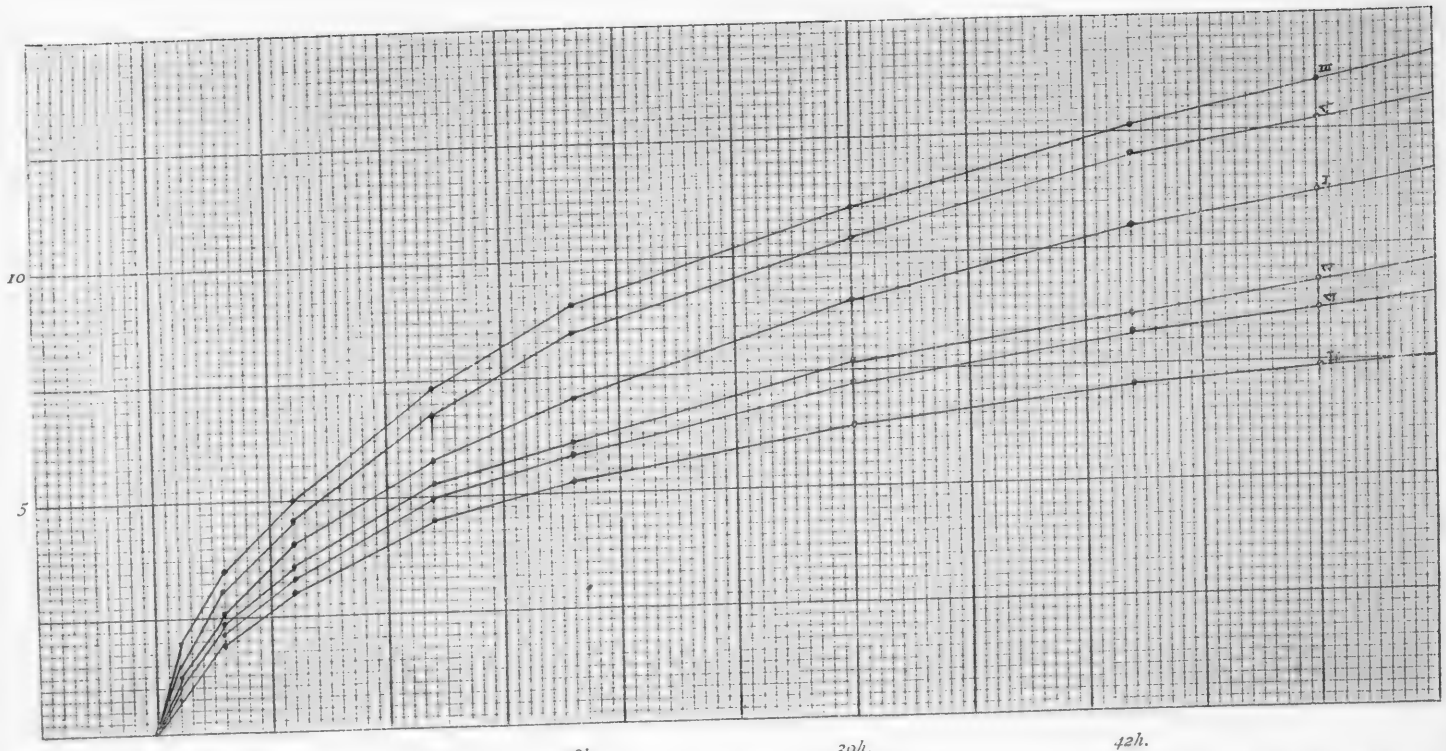
The experiments with $\frac{1}{100}$ N. saponin dissolved in agar and dog blood suspended in gelatin showed diffusion to take place more rapidly into 10 than into 25 per cent. gelatin, and much more rapidly from agar than from the saline control. When, however, the colloids were reversed, namely, the blood suspended in agar and the solanin desolved in gelatin, the influence of the concentration of the gelatin came out, the diffusion from the saline being greater than that from the colloid. Plates XXVII and XXVIII express these effects in the form of curves.

The explanation of this discrepancy is to be found, possibly, in the sum of inhibitions exerted by the gelatin and the corpuscles, of which the first is the greater; while, as has frequently appeared in these experiments, the greatly weaker agar concentrations exercise slight inhibition only. A similar effect, although a smaller one, is produced in the case of pyrogallic acid.

In the study of the more complex hæmolysins of venom it was found, in spite of certain irregularities of diffusion, that (1) the velocity of diffusion from agar into the gelatin blood is slower in the case of 25 than in the case of 10 per cent. gelatin, (2) the hæmolysin of cobra venom is diffused from agar into gelatine more slowly than from these colloids into saline, (3) concentrations of agar between 0.5 and 2.0 per cent. act about the same, and (4) the relation of time to diffusion does not correspond to Fick's law.



I From saline into 0.5% agar.
II From saline into 1% agar.
III From 10% gelatin into 1% agar.
IV From 25% gelatin into 1% agar.



I From saline into 10% gelatin.
II From saline into 25% gelatin.
III From 0.5% agar into 10% gelatin.
IV From 0.5% agar into 25% gelatin.
V From 1% agar into 10% gelatin.
VI From 1% agar into 25% gelatin.

DIFFUSION FROM COLLOID INTO SALINE SOLUTION.

Sodium taurocholate, saponin, and solanin were dissolved in 10 and 25 per cent. gelatin and poured into test-tubes of 20 mm. diameter. Five cubic centimeters of the colloids were put into each tube after the congealation of which an equal quantity of saline solution was poured in. The saline fluid, after contact, was removed at stated periods, and the quantity of hæmolysin present in it was estimated colorimetrically. Three

TABLE I.

SAPONIN $\frac{1}{100}$ N.

Period of Contact	Degree of Hæmolysis	25% gelatin		10% gelatin	
		Quantity in c.c. required to produce stated degree of hæmolysis	Corresponding number of hæmolytic units in 1 c.c. of saline	Quantity in c.c. required to produce stated degree of hæmolysis	Corresponding number of hæmolytic units in 1 c.c. of saline
1 hour	100%				
	30%	1.8	0.55	1.	1.
	5%	0.9	1.1	0.55	1.8
2 hours	100%				
	30%	1.4	0.7	0.6	1.6
	5%	0.7	1.4	0.5	2.
4 hours	100%			1.	1.
	30%	0.75	1.33	0.5	2.
	5%	0.5	2.	0.35	2.8
24 hours	100%	0.7	1.4	0.5	2.
	30%	0.3	3.3	0.2	5.
	5%	0.18	5.5	0.14	7.1

SOLANIN $\frac{1}{100}$ N.

1 hour	100%	0.55	1.8	0.33	3.
	30%	0.14	7.	0.09	11.
	5%	0.125	8.	0.085	12.
2 hours	100%	0.33	3.	0.25	4.
	30%	0.09	11.	0.075	13.3
	5%	0.085	12.	0.07	14.
4 hours	100%	0.25	4.	0.18	5.5
	30%	0.07	14.	0.055	18.
	5%	0.055	18.	0.04	25.
24 hours	100%	0.1	10.	0.07	14.2
	30%	0.03	33.	0.025	40.
	5%	0.025	40.	0.02	50.

grades were used: 100, 30, and 5 per cent. It was found that sodium taurocholate was largely held back for at least 48 hours by the gelatin, probably because of a chemical union between the salt and the proteid. Only a trace of hæmolysis was obtained at the end of this period. The results obtained with saponin and solanin are given in the accompanying table (Table I), and they show that the quantities of these bodies diffused from 25 and 10 per cent. gelatin bear the proportion of 1 : 1.25–1.40. *Hence the velocity and amount of diffusion from colloid into saline, like that from saline into colloid, is inversely proportional to the square root of the concentration of the colloid.*

INFLUENCE UPON THE RATE OF DIFFUSION OF DIAMETER OF SURFACE, DEPTH OF MEDIUM, AND CONCENTRATION OF HÆMOLYSIN.

TABLE II.

Diameter of Tube	Amount of Gelatin Blood	Amount of Saponin Solution	Period of Contact	$\frac{1}{100}$ N. Saponin 25% gelatin	$\frac{1}{100}$ N. Saponin 10% gelatin	$\frac{1}{1000}$ N. Saponin 25% gelatin	$\frac{1}{1000}$ N. Saponin 10% gelatin	$\frac{1}{10000}$ N. Saponin 25% gelatin	$\frac{1}{10000}$ N. Saponin 10% gelatin
8 mm.	4c.c	4c.c.	6 hours	2.4	2.85		1.9		
			24 "	5.	6.	4.2	4.55	1.75	1.75
			48 "	7.	8.4	6.45	7.35	3.85	3.85
	4 c.c.	2 c.c.	6 hours	2.4	2.85		1.9		
			24 "	5.	6.	4.2	4.55	1.75	1.75
			48 "	7.	8.4	6.45	7.35	3.85	3.85
	2 c.c.	4 c.c.	6 hours	2.4	2.85		1.9		
			24 "	5.	6.	4.2	4.55	1.75	1.75
			48 "	7.	8.4	6.45	7.35	3.85	3.85
15 mm.	2 c.c.	2 c.c.	6 hours	2.4	2.85	>			
			24 "	5.	6.	4.2	4.55	2.	2.1
			48 "	7.	8.75	7.	7.7	3.85	3.85
	2 c.c.	2 c.c.	6 hours	2.4	2.85	>			
			24 "	5.	6.	4.2	4.55	2.	2.1
			48 "	7.	8.75	7.	7.7	3.85	3.85
	1 c.c.	2 c.c.	6 hours	2.4	2.85	>			
			24 "	5.	6.	4.2	4.55	2.	2.1
			48 "	7.5	9.1	7.	7.7	3.85	3.85

The substances used in these experiments were solanin and saponin in saline solution and dog blood mixed with gelatin. The results are given in Tables II and III and they show that no marked influence is exercised on the diffusion by diameter of surface and depth of medium. But other facts may be noted.

TABLE III.

Diameter of Tube	Amount of Gelatin Blood	Amount of Solanin Solution	Period of Contact	$\frac{1}{100}$ N. Solanin 25% gelatin	$\frac{1}{100}$ N. Solanin 10% gelatin	$\frac{1}{1000}$ N. Solanin 25% gelatin	$\frac{1}{1000}$ N. Solanin 10% gelatin	$\frac{1}{10000}$ N. Solanin 25% gelatin	$\frac{1}{10000}$ N. Solanin 10% gelatin
15 mm.	4 c.c.	4 c.c.	6 hours	3.6	4.6		1.25	Slight trace in 48 hours.	ditto.
			24 "	7.5	9.5	1.4	3.85		
			48 "	10.5	13.5	2.1	5.95		
	4 c.c.	2 c.c.	6 hours	3.6	4.6		1.25		
			24 "	7.5	9.5	1.3	3.85		
			48 "	10.5	13.5	2.1	6.		
	2 c.c.	4 c.c.	6 hours	3.6	4.6		1.25		
			24 "	7.5	9.5	1.4	3.85		
			48 "	10.5	13.5	2.1	5.95		
8 mm.	2 c.c.	2 c.c.	6 hours	3.6	4.6		1.25	Slight trace in 48 hours.	ditto.
			24 "	7.5	9.8	1.4	3.85		
			48 "	10.5	13.5	2.1	6.		
	2 c.c.	1 c.c.	6 hours	3.6	4.6		1.25		
			24 "	7.5	9.8	1.4	3.85		
			48 "	10.5	13.5	2.1	5.9		
	1 c.c.	2 c.c.	6 hours	3.6	4.6		1.25		
			24 "	8.	9.8	1.4	3.85		
			48 "	10.5	13.5	2.1	6.		

The relative capacity for diffusion of saponin and solanin from saline solution into gelatin blood is expressed in hæmolytic units by the proportion of 1:1.5. These figures are to be compared with those in which the hæmolysin is enclosed in the colloid (Table I) and allowed to diffuse into saline, in which series the solanin is shown to diffuse from seven to eight times more hæmolytic units in a given period than the saponin. But

as solanin is nearly twice as strong an hæmolytic agent as saponin the proportion of diffused hæmolytic units would stand, as regards diffusion from colloid into saline, as 1:4 against 1:1.5 for saline into colloid. The diffusion velocity for solanin is, under the former conditions, four times as great as for saponin; and the inhibiting influence of the colloid is greater when the crystalloid seeks to enter it from than when it attempts to leave it for a fluid medium.

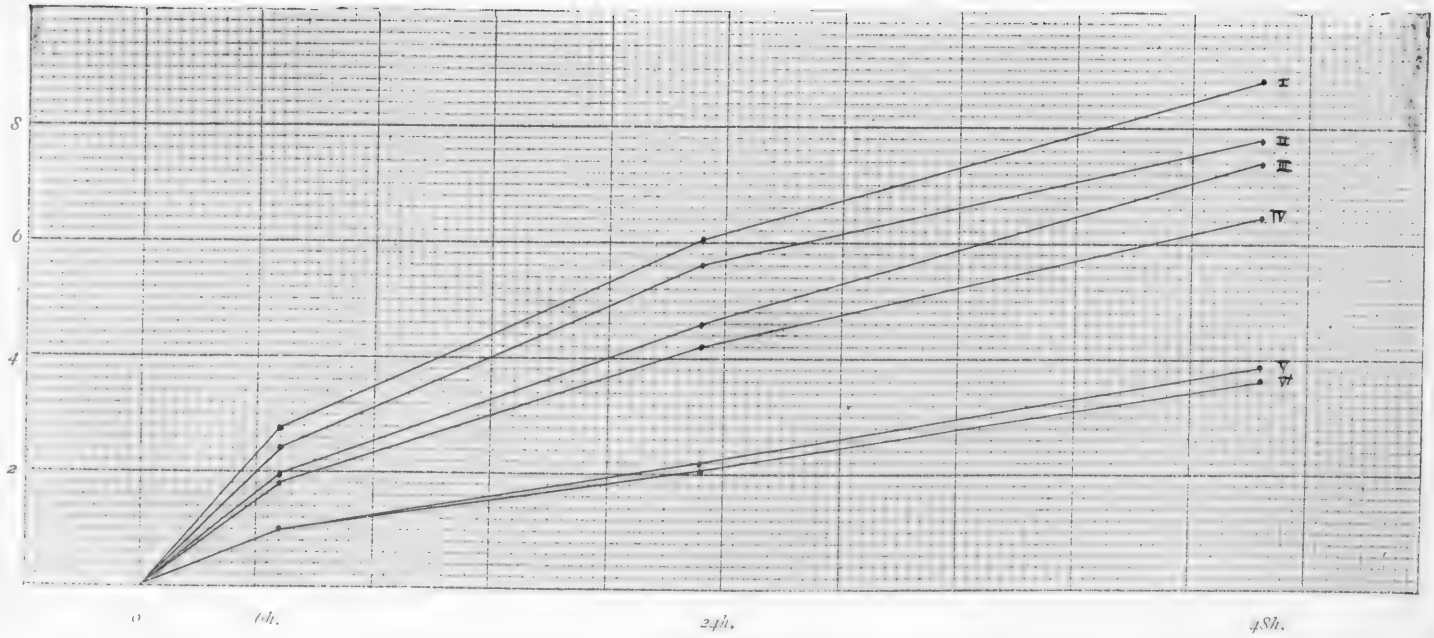
The concentration of the active substance plays a considerable part in relation to the rate of diffusion. The effects of this concentration are best seen by observing Plates XXIX and XXX. In the case of saponin while the difference in diffusion between $\frac{1}{100}$ N. and $\frac{1}{1000}$ N. is very slight, that between $\frac{1}{1000}$ N. and $\frac{1}{10000}$ N. becomes as about 2:1. In the case of solanin the difference between $\frac{1}{100}$ N. and $\frac{1}{1000}$ N. is as 2:1, and between $\frac{1}{1000}$ N. and $\frac{1}{10000}$ N. more than 10:1.

In Plate XXIX, the following values are given: I and II represent the degree of diffusion of $\frac{1}{100}$ N. saponin from saline into 10 per cent. and 25 per cent. gelatin respectively; III and IV represent the degree of diffusion of $\frac{1}{1000}$ N., and V and VI, the degree of diffusion of $\frac{1}{10000}$ N. saponin under the same conditions. The values for Plate XXX are as follows: I and II represent the degree of diffusion of $\frac{1}{100}$ N. solanin from saline into 10 per cent. and 25 per cent. gelatin respectively; and III and IV the degree of diffusion of $\frac{1}{1000}$ N. solanin under the same conditions.

TABLE IV.

Scale of Hæmolysis*	Saponin				Solanin			
	25% gelatin		10% gelatin		25% gelatin		10% gelatin	
	$\frac{1}{100}$ N	$\frac{1}{1000}$ N	$\frac{1}{100}$ N	$\frac{1}{1000}$ N	$\frac{1}{100}$ N	$\frac{1}{1000}$ N	$\frac{1}{100}$ N	$\frac{1}{1000}$ N
a	1.		0.6		0.25	2.	0.16	1.5
b	0.3		0.25		0.06	0.7	0.05	0.5
c	0.2	2.	0.15	1.5	0.045	0.5	0.035	0.35

*The scale of hæmolysis was an arbitrary and descending one—a being highest and c lowest.



Saponin.

1/100 N. Solution { I From saline into 10% gelatin. / II From saline into 25% gelatin.

1/1000 N. Solution { III From saline into 10% gelatin. / IV From saline into 25% gelatin.

1/10000 N. Solution { V From saline into 10% gelatin. / VI From saline into 25% gelatin.

I
II
III
IV
12
10
8
6
4
2
0
6h.
24h.
48h.
Solanin.
1/100 N. Solution { I From saline into 10% gelatin.
II From saline into 25% gelatin.
1/1000 N. Solution { III From saline into 10% gelatin.
IV From saline into 25% gelatin.

TABLE V.

Period of Contact	Degree of Hæmolysis	$\frac{1}{100}$ N. Solanin		$\frac{1}{1000}$ N. Solanin	
		25% gelatin	10% gelatin	25% gelatin	10% gelatin
1 hour	30%	0.14*	0.09	1.5	1.
	5%	0.125	0.085	1.	0.65
2 hours	30%	0.09	0.075	1.	0.8
	5%	0.085	0.07	0.7	0.55
4 hours	30%	0.07	0.055	0.7	0.6
	5%	0.05	0.04	0.5	0.4
24 hours	30%	0.03	0.025	0.03	0.25
	5%	0.025	0.02	0.25	0.2

* These figures indicate the quantity of supernatant saline required to produce the degree of hæmolysis stated.

The differences noted are not entirely easy of explanation; and it is especially difficult to account for the behavior of solanin, in that while diffusing much more readily than saponin, it should, at the same time, be so greatly influenced by concentration. These considerations apply only to the special case in which the hæmolysins are contained in the saline; when they are enclosed in the colloid and allowed to diffuse into saline the amount of diffusion is directly proportional to the concentration of the hæmolysins.

DIFFUSION OF COBRA VENOM AND TETANUS TOXIN INTO COLLOIDS.

It has been stated that the hæmolysins of cobra venom and tetanus toxin pass from saline solution into gelatin and agar. A series of experiments was made to determine the effect of enclosing the toxins in one colloid (agar) and the blood in another (gelatin), and the reverse. Using a 5 per cent. suspension of dog blood in 10 per cent. gelatin and 0.1 per cent. cobra venom

in 2 per cent. agar, it was found that complete hæmolysis occurred in twenty-four hours, while when the blood was mixed with the agar and the venom with the gelatin, the hæmolysis proceeded much more slowly. It was also ascertained that the venom hæmolysin passes more quickly into gelatin from agar than from saline solution. In this respect it agrees with the more definite chemical hæmolysins.

The next question considered was whether the entire series of venom principles pass into the colloids, and if so whether they diffuse with equal velocity. To test this the hæmolysed portion of the gelatin- and agar-blood mixtures was carefully separated from the other in which the blood corpuscles were still intact, and each portion was injected into guinea-pigs to determine the toxicity. In the case of the gelatin-blood mixture the portion to be injected intraperitoneally was first melted, and in the case of agar it was emulsified in saline solution. The gelatin used was 10 per cent., and the agar 2 per cent. for enclosing the venom, and 0.5 per cent. for enclosing the blood.

Two cubic centimeters of the venom-colloid mixture were used in each experiment. The number of minimal lethal doses contained in this quantity, for guinea-pigs, of about 180 grams weight, was sixteen. It was ascertained that three m.l.d. of the venom passed in the hæmolysed portion from the agar into the gelatin blood, and one m.l.d. from the gelatin into the agar blood. It was further ascertained that the non-hæmolysed portion of the colloid-blood mixture does not contain the cobra neurotoxin in appreciable quantities. In other words, cobra lysin and cobra neurotoxin diffuse together.

Since the two principles diffuse together it was desirable to ascertain, if possible, whether they diffuse accurately in the proportion in which they exist in venom; that is, whether the whole venom passes unchanged into the colloid, or whether a separation of the components occurs. It has been stated that, using two cubic centimeters of a 0.1 per cent. cobra venom suspension in agar, one fifth of its toxic constituents passed into the gelatin-blood mixture in twenty-four hours. It was ascertained that two cubic centimeters of the venom mixture contained 600

complete hæmolytic doses of cobra lysin (for dog blood). Since the rate of diffusion of cobra lysin is very little affected by time, as it proceeds almost in a straight line, all that is required to determine the quantity of diffused lysin is to multiply the amount of diffusion by the time. It was found by experiment that an amount of cobra lysin necessary to hæmolyse two cubic centimeters of a 5 per cent. suspension of dog corpuscles will require from three to four hours to pass from 10 per cent. gelatin containing 0.1 per cent. venom to saline solution; and that in twenty-four hours about twenty-four complete hæmolytic doses will have diffused into the saline. Hence the rate of diffusion of cobra lysin is as 24: 600 or 1:25. In the same time, as we have seen, the quantity of cobra neurotoxin which diffused from agar into gelatin was as 1:5. In other words, cobra neurotoxin diffused five times faster than cobra lysin from agar into a gelatin-blood mixture. This fact interesting in itself is rendered more suggestive in view of Faust's [11] recent statement of a relationship between cobra neurotoxin and the saponin substances.

A series of experiments similar to the above was made with a tetanus toxin one cubic centimeter of which contained 1000 minimal lethal doses for guinea-pigs of 250 grams and rats of 60 grams weight, and 33 minimal hæmolytic doses. It was found that both tetanolysin and tetanospasmin pass together easily into gelatin and agar, since the non-hæmolysed portions of the blood-colloid cylinder do not contain an appreciable quantity (less than 0.0001 c.c.) of the toxin. It would, however, appear from our experiments that tetanolysin diffuses more rapidly into colloids than tetanospasmin, since the great disproportion in quantity of spasmin and lysin—1000:33—which exists in the tetanus toxin does not bring about a corresponding preponderance of the spasmin in the colloid.

SUMMARY.

Acids, alkalies, salts, glucosides, and certain toxins diffuse

[11] Die tierische Gifte, Braunschweig, 1906, p. 54.

more quickly into 0.9 per cent. salt solution than into agar-agar and gelatin suspensions.

The inhibitory effect of the colloids grows with increase in concentration, which increase affects both the velocity and extent of the diffusion.

In the case of gelatin the degree of diffusion is approximately in inverse proportion to the square root of the concentration. Agar-agar in strengths up to 2 per cent. inhibits far less than gelatin in 10 per cent. suspensions; and the difference in degree of inhibition exercised by 0.5 per cent. and 2 per cent. agar-agar is a small one.

Hæmolytic substances diffuse from gelatin into agar-agar more slowly than from saline into agar-agar. But the velocity of diffusion from agar-agar into gelatin is greater than from saline into gelatin.

The effects of differences in concentration of the hæmolytic agent vary according to the agent and the manner of its solution. When the hæmolyser is dissolved in salt solution the diffusion of $\frac{1}{100}$ N. and $\frac{1}{1000}$ N. solutions (saponin) is almost identical; while with solanin the stronger solutions diffuse faster. When the hæmolyser is dissolved in the colloid diffusion into fluid media is nearly proportional to the concentrations of the hæmolytic agent.

The velocity of diffusion into and from colloids is in general proportional to the square root of the time. Acids, alkalies, salts, and glucosides act in a manner which is in agreement with this rule. Cobra lysin and tetanolysin do not act in conformity with the rule.

Cobra lysin appears to diffuse into colloids more slowly, proportionally, than cobra neurotoxin, and tetanospasmin more slowly than tetanolysin.

The biological method described in this paper for studying diffusion in colloids is applicable to hæmolytic and some other toxic substances, and, with accuracy possibly only to such substances possessing relatively simple compositions.

Since all diffusion in the living body takes place within colloidal media of different concentrations, it would seem desirable to

perfect methods through which the interaction of toxic chemicals and the fluids and cells of the body may in a manner be imitated *in vitro*. Through this means our knowledge of toxicology may well be extended.